Landmark Article
Oct 31, 1936
(*JAMA* 1936;107:1456-1463)

Observations on the Etiologic Relationship of Achylia Gastrica to Pernicious Anemia

V. Further Evidence for the Essential Participation of Extrinsic Factor in Hematopoietic Responses to Mixtures of Beef Muscle and Gastric Juice and to Hog Stomach Mucosa

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Observations on patients with addisonian pernicious anemia have appeared to us to demonstrate that the immediate basis of the anemia is a "conditioned" defect of nutrition. Thus, patients suffering from pernicious anemia are seemingly unable to derive from food some substance essential for normal function of bone marrow. The nutritional defect in such patients is apparently caused by the failure of a reaction which occurs in the normal individual between a substance in the food (extrinsic factor) and a substance in the normal gastric secretion (intrinsic factor). This conclusion is based on the following evidence derived from previous observations[1] on cases of addisonian pernicious anemia:

1. The daily administration of (extrinsic factor) 200 Gm. of beef muscle is without significant effect on blood formation.

2. The daily administration of (intrinsic factor) from 150 to 300 cc. of normal human gastric juice is without significant effect on blood formation.

3. If, however, such amounts of each substance are administered daily in such a way as to permit contact either before or after administration to the patient, clinical improvement and evidence of increased blood formation are usually apparent within ten days and are progressive for the duration of such therapy.

Apparent confirmation of these basic observations has

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The expenses of this investigation were defrayed in part by a grant from the Proctor Fund of Harvard University for the Study of Chronic Disease and by the J. K. Lilly Gift to the Harvard Medical School.

Owing to lack of space, this article has been abbreviated in THE JOURNAL by the omission of a section of the text and corresponding bibliography. The complete paper will appear in the authors' reprints.

The observations on certain patients were made possible through the kind cooperation of members of the staff of the First and Third Medical Services (Tufts) of the Boston City Hospital. We are indebted to Miss Margaret Evans and to Miss Eleanor Fleming for assistance in performing the blood studies.

1. These include:

(*a*) Castle, W. B.: Observations on the Etiologic Relationship of Achylia Gastrica to Pernicious Anemia: I. The Effect of Administration to Patients with Pernicious Anemia of the Contents of the Normal Human Stomach Recovered After the Ingestion of Beef Muscle, Am. J. M. Sc. **178**:748 (Dec.) 1929.

(*b*) Castle, W. B., and Townsend, W. C.: Observations on the Etiologic Relationship of Achylia Gastrica to Pernicious Anemia: II. The Effect of the Administration to Patients with Pernicious Anemia of Beef Muscle After Incubation with Normal Human Gastric Juice, ibid. **178**:764 (Dec.) 1929.

(*c*) Castle, W. B., Townsend, W. C., and Heath, C. W.: Observations on the Etiologic Relationship of Achylia Gastrica to Pernicious Anemia: III. The Nature of the Reaction Between Normal Human Gastric Juice and Beef Muscle Leading to Clinical Improvement and Increased Blood Formation Similar to the Effect of Liver Feeding, ibid. **180**:305 (Sept.) 1930.

(*d*) Castle, W. B., Heath, C. W., and Strauss, M. B.: Observations on the Etiologic Relationship of Achylia Gastrica to Pernicious Anemia: IV. A Biologic Assay of the Gastric Secretion of Patients with Pernicious Anemia Having Free Hydrochloric Acid and That of Patients Without Anemia or with Hypochromic Anemia Having No Free Hydrochloric Acid, and of the Role of Intestinal Impermeability to Hematopoietic Substances in Pernicious Anemia, ibid. **182**:741 (Dec.) 1931.

(*e*) Strauss, M. B., and Castle, W. B.: The Nature of the Extrinsic Factor of the Deficiency State in Pernicious Anemia and in Related Macrocytic Anemias: Activation of Yeast Derivatives with Normal Human Gastric Juice, New England J. Med. **207**:55 (July 14) 1932.

been obtained by various workers, notably Groen,[2] Hartfall and Witts,[3] Helmer, Fouts and Zerfas,[4] Middleton and Stiehm,[5] Miller and Rhoads,[6] Reimann,[7] Singer,[8] Ungley and James[9] and Wilkinson and Klein,[10] who have agreed with us in supposing the necessity of both an extrinsic and an intrinsic factor for such increased blood production in pernicious anemia. In a recent paper, however, Greenspon[11] has presented the results of experiments from which he has drawn the conclusion that a food (extrinsic) factor is unessential to the production of the positive effects on blood formation in pernicious anemia which we reported from incubated mixtures of beef muscle and gastric juice. He believes that our negative results with normal human gastric juice alone were due to destruction of an "antipernicious anemia principle" by peptic action during the preliminary incubation usually employed. As a corollary, it was inferred by Greenspon that when gastric juice was incubated with beef muscle "the native pepsin in the gastric juice must have been adsorbed by the ground beef" and thus "the beef served to protect the antipernicious anemia principle and not as a substrate for the action of an enzyme-like 'intrinsic factor.'"

Previous evidence exists, however, which would appear to render Greenspon's conclusions unlikely. In our former observations[1b] on patient 11, gastric juice which was not incubated before administration yielded negative results. Middleton and Stiehm[5] and also Groen[2] have obtained similar negative results. Nevertheless, according to Greenspon's theory the activity of such unneutralized gastric juice might have been destroyed by peptic hydrolysis in vivo after administration to the patient. Therefore these observations are not necessarily conclusive. The experiments of Helmer, Fouts and Zerfas[4] can scarcely be so criticized, however, since the gastric juice employed by them was depepsinized and brought to a p_H of 4.7 to 5 before administration. In case 8 of their series the daily administration of 150 cc. of such gastric juice was ineffective.

It is thus not certain that Greenspon's experiments throw doubt on the conclusions that we have drawn. Furthermore, for reasons that will be presented later, it seems to us that, unless the necessity for a food factor is conceded, our observations as well as his own do not necessarily disclose the immediate etiologic mechanism in addisonian pernicious anemia. Moreover, without invoking a food factor, it is difficult to find a ready explanation of the etiologic relationship to pernicious anemia of certain other types of macrocytic anemia which likewise respond to the administration of liver or stomach preparations. For these reasons, a repetition of certain of Greenspon's experiments was undertaken as well as a critical analysis of our former observations.

METHODS

The ten patients included in the present observations were all typical cases of addisonian pernicious anemia. Each had gastric anacidity and an initial red blood cell count of less than 2 million per cubic millimeter. In distinguishing between negative and positive effects on blood formation, use was made of the reticulocyte response that occurs with positive effects on blood formation in suitable patients with pernicious anemia. For this purpose the reticulocyte response was used in all our former observations as well as in Greenspon's[11] experiments. A full discussion of the significance of such reticulocyte responses has recently been published.[12] The methods of blood counting and of reticulocyte staining were those employed in our previous studies. Unless otherwise specified, the normal human gastric juice (150 cc.) was secreted by a healthy fasting individual after the injection of 0.5 mg. of histamine, was then filtered through coarse cloth and placed in the icebox. The patients were maintained on the basal diets used in former observations, which contained no meat, eggs, liver or kidney. Chicken and fish were allowed once or twice a week. In cases 62, 63, 64, 66, 68 and 69 the basal diet was further restricted during the periods of observation and consisted of white bread, rice, macaroni, butter, potato, ice cream, tea, coffee and sugar.

OBSERVATIONS

Normal human gastric juice does not contain an "antipernicious anemia principle" effective on oral administration without contact with food (extrinsic) factor.

As already pointed out, Greenspon[11] does not share this view but considers that gastric juice contains an "antipernicious anemia principle" effective when fed alone. He bases his belief partly on the following direct experimental evidence:

Two normal subjects, after having been given 60 grains (4 Gm.) of calcium carbonate orally as a neutralizing agent, were injected with histamine in order to stimulate the flow of gastric

2. Groen, Juda: Klinisch en experimenteel onderzoek over anaemia perniciosa en voorwaardelijke deficientie, Amsterdam, Scheltema & Kolkema's Boekhandel, 1935.

3. Hartfall, St. J., and Witts, L. J.: The Intrinsic Factor of Castle in Simple Achlorhydric Anaemia, Guy's Hosp. Rep. 83:24 (Jan.) 1933.

4. Helmer, O. M.; Fouts, P. J., and Zerfas, L. G.: Relationship of Intrinsic Factor to Hematopoietic Material in Concentrated Human Gastric Juice, Am. J. M. Sc. 188:184 (Aug.) 1934.

5. Middleton, W. S., and Stiehm, R. H.: The influence of Gastric Juice on Erythropoiesis in Pernicious Anemia, Am. J. M. Sc. 180:809 (Dec.) 1930.

6. Miller, D. K., and Rhoads, C. P.: The Presence in Egg-White and in Rice-Polishings Concentrate Low in Vitamin B_2 (G) of an Anti-Pernicious Anemia Principle, New England J. Med. 211:921 (Nov. 15) 1934.

7. Reimann, F.: Zur Frage der Steigerung der antianämischen Wirkung der Leber durch die Einwirkung von Magensaft auf Leber, Klin. Wchnschr. 13:413 (March 17) 1934.

8. Singer, K.: Eiertherapie der perniziösen Anämie, Wien. klin. Wchnschr. 45:1063 (Aug. 26) 1932.

9. Ungley, C. C., and James, G. V.: The Effect of Yeast and Wheat Embryo in Anaemias: II. The Nature of the Haemopoietic Factor in Yeast Effective in Pernicious Anaemia, Quart. J. Med. 27:523 (Oct.) 1934.

10. Wilkinson, J. F., and Klein, L.: The Active Principle in Hog's Stomach Effective in Pernicious Anaemia, Lancet 1:719 (April 2) 1932; The Relationship Between the Antianaemic Principles in Stomach and Liver, ibid. 2:629 (Sept. 16) 1933.

11. Greenspon, E. A.: The Nature of the Antipernicious Anemia Principle in Stomach: I. Method to Improve Stomach Preparations, J. A. M. A. 106:266 (Jan. 25) 1936.

12. Minot, G. R., and Castle, W. B.: The Interpretation of Reticulocyte Reactions: Their Value in Determining the Potency of Therapeutic Materials, Especially in Pernicious Anaemia, Lancet 2:319 (Aug. 10) 1935.

TABLE 1.—*Negative Results of the Administration to Patients with Pernicious Anemia of Neutralized Gastric Juice (Greenspon), and of Gastric Juice and Beef Muscle Administered Without Opportunity for Contact; Positive Effects of Gastric Juice (Before or After Incubation at 37.5 C. for Two Hours) Administered with Beef Muscle, and of Gastric Juice Administered with Previously Inactivated Hog Stomach Mucosa*

First Periods.—Daily Administration of Various Substances Except as Indicated Below

	Gastric Juice 250 Cc. Neutralized with Calcium Carbonate (Greenspon)						Gastric Juice 250 Cc. Incubated 2 Hrs. p_H 1.5, Then to p_H 7.0, with Beef Muscle 200 Gm.		Hog Stomach Mucosa 200 Gm. Incubated 48 Hrs. p_H 3.0, Then to p_H 5.5		Gastric Juice 150 Cc. p_H 7.0		Gastric Juice 150 Cc. Mixed with Boiled Hog Stomach Mucosa 200 Gm. p_H 7.0		Beef Muscle 300 Gm. and Gastric Juice 150 Cc. p_H 7.0 Respectively, at 8 A.M. on Alternate Days		Beef Muscle 200 Gm. at 8 A.M.; Gastric Juice 150 Cc. p_H 7.0 at 8 P.M.		Beef Muscle 200 Gm. at 10 A.M.: Gastric Juice 100 Cc. p_H 7.0 at 4 P.M.	
	Case 62		Case 63		Case 64		Case 65		Case 7a		Case 66		Case 67		Case 68		Case 69		Case 70	
Days of Treatment	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent
0	1.73	2.4	1.77	1.6	0.82	0.4	0.89	1.4	1.52	0.8	1.86	1.4	0.82	1.4	1.84	1.4	1.60	3.4	1.57	0.9
2	1.94	1.9	1.70	1.7	....	1.0	0.89	1.4	1.56	2.8	1.97	1.4	0.74	1.6	1.57	1.2	1.53	3.2	1.90	3.5
4	1.81	0.6	1.70	1.6	0.94	0.2	0.85	3.2	1.49	2.0	1.98	3.4	0.70	1.0	1.80	0.4	1.44	2.8	1.65	2.2
6	1.75	0.5	1.57	0.4	....	0.8	1.18	13.5	1.41	2.4	1.93	2.6	0.72	3.1	1.84	1.2	1.49	3.8	1.94	3.0
8	1.73	0.3	1.56	0.4	0.98	0.6	1.26	14.5	1.63	2.2	2.18	2.6	0.81	13.2	1.68	1.0	1.70	4.0	1.99	2.6
10	1.67	0.4	1.55	1.0	0.71	2.1	1.45	12.8	1.58	1.6	1.98	0.9	1.10	29.0	1.64	1.2	1.42	2.8	2.03	4.0
12	....	.....	....	.....	0.97	1.9	1.67	11.0	1.33	1.2	2.04	2.0	....	.....	1.68	1.4	1.34	1.4	2.16	9.0
14	....	.....	....	.....	....	1.6*	....	.....	1.41	0.8	....	.....	....	.....	....	.....	....	.....	2.45	4.6

Second Periods.—Daily Administration of Various Substances Except as Indicated Below

	Gastric Juice 250 Cc. Incubated 1 Hr. p_H 1.5, Then to p_H 7.0, with Beef Muscle 200 Gm.		Gastric Juice 250 Cc. Incubated 2 Hr. p_H 1.5, Then to p_H 7.0, with Beef Muscle 200 Gm.		Continued as in First Period		Gastric Juice 250 Cc. p_H 7.0, with Beef Muscle 200 Gm.		Liver Extract-Lilly from 600 Gm. Liver		Gastric Juice 150 Cc. Mixed with Boiled Hog Stomach Mucosa 200 Gm. p_H 7.0		No Therapy		Gastric Juice 150 Cc. p_H 7.0 and Beef Muscle 300 Gm. Together on Alternate Days		Gastric Juice 150 Cc. p_H 7.0 and Beef Muscle 200 Gm. Together	
	Case 62		Case 63		Case 64		Case 65		Case 7a		Case 66		Case 67		Case 68		Case 69	
	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent
2	1.72	0.6	1.47	0.6	1.28	1.9	1.74	6.4	1.07	0.6	2.04	1.0	1.31	35.9	1.53	1.6	1.46	3.0
4	1.73	0.2	1.34	1.2	1.39	1.0	1.52	4.4	1.17	5.4	2.04	0.2	1.35	18.2	1.35	1.0	1.43	2.8
6	1.84	0.9	1.44	2.1	....	0.8	1.74	8.7	....	18.2	2.13	0.2	1.45	5.4	1.52	4.0	1.47	7.1
8	1.74	2.9	1.43	5.6	1.42	1.1	1.88	13.9	1.97	20.4	2.07	4.2	1.69	3.1	1.53	5.0	1.55	8.4
10	1.94	9.3	1.61	9.5	1.09	1.5	2.32	15.9	2.31	26.0	2.17	7.5	1.85	4.3	1.30	5.9	1.52	20.6
12	....	.....	1.75	12.3	....	1.3	2.47	5.6	2.64	5.2	2.04	3.8	....	.....	1.55	6.5	1.68	13.0
14	....	.....	1.95	8.4	1.10	0.8	....	.....	....	.....	2.28 (Nonprotein nitrogen 73.8 mg.)	2.9						

Third Periods.—Daily Administration of Various Substances as Indicated Below

	No Therapy		Gastric Juice 250 Cc. p_H 7.0, with Beef Muscle 200 Gm.		Ventriculin 10 Gm.										No Therapy		No Therapy	
	Case 62		Case 63		Case 64		Case 65		Case 7a		Case 66		Case 67		Case 68		Case 69	
	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent	Red Blood Cells, Millions	Reticulocytes, per Cent
2	2.33	16.1	1.80	4.4	....	0.9	....	.....	....	.....	....	.....	....	.....	....	5.0	1.86	10.2
4	2.67	7.1	1.75	1.8	0.91	5.0	....	.....	....	.....	....	.....	....	.....	1.47 (Cystitis)	7.4	1.95	3.8
6	2.70	2.9	1.60	2.2	0.97	7.0												
8	....	.....	1.83	6.8	....	10.1												
10	....	.....	1.80	14.5	0.74	14.8												
12	....	.....	2.07	10.6	....	10.4												
14	....	.....	1.98	9.0	0.67	7.4												
16	....	.....	2.16	3.0	0.83 (10 Days Later)	5.4												
					1.40	5.0												

*Transfusion of 250 cc. of blood.

juice. By means of a Rehfuss tube the gastric juice was then aspirated and collected in a glass beaker containing ice and surrounded by ice. Care was taken immediately to adjust the reaction of the juice to neutrality and to maintain it so, until it was given to a pernicious anemia patient who had been selected for the testing of this material. The patient was fed about 250 cc. of this cold, neutralized gastric juice each day. It was given in the morning, on an empty stomach, and no food was allowed for the following four hours, in order to avoid the introduction of the so-called extrinsic factor.

In one patient with pernicious anemia and an initial red blood cell level of 2.6 million per cubic millimeter, Greenspon found a reticulocyte peak of 14 per cent on the seventh day of this regimen.

The technic of Greenspon's experiment was exactly followed in observations on patients 62, 63 and 64[13] except that no ice was put into the gastric juice, exactly 250 cc. was administered, and the time interval before food was lengthened to six hours. Patient 66 was given daily 150 cc. of normal human gastric juice neutralized only immediately before administration. No significant effect on reticulocyte or red blood cell production was observed during periods of ten days in patients 62, 63 and 66, and during a period of twenty-eight days in patient 64 (table 1). None of the patients were clinically improved and the condition of patient 64 necessitated a transfusion of 250 cc. of blood on the fourteenth day. Similar negative results from observations with gastric juice neutralized by the method of Greenspon have recently been reported by Flood and West,[14] Hanes, Hansen-Prüss and Edwards,[15] Ungley and Moffett,[16] and Fitz-Hugh and Creskoff.[16a]

The immediately succeeding period of observation in cases 62, 63, 64 and 66 demonstrates the ability of each patient to react positively. Patients 62 and 63 were given a meal containing 200 Gm. of beef muscle simultaneously with 250 cc. of gastric juice unneutralized until immediately before administration at noon each day. Patient 64 responded to the daily administration of 10 Gm. of ventriculin, as did patient 66 to the administration of a mixture of gastric juice and boiled hog stomach mucosa. The details of the blood counts are presented in table 1. A consideration of the nature of the diet given Greenspon's patient suggests the probable explanation for his isolated positive result with neutralized gastric juice fed alone. Since meat,[1b] eggs[8] and whole grain cereals[17] have been shown to yield positive results when administered with gastric juice to patients with pernicious anemia, patients 62, 63, 64 and 66 were given none of these foods in the special basal diet, specified under methods. Dr. Greenspon has kindly informed us that, on the contrary, the only dietary restriction imposed on his patient was the omission of liver and kidney. The patient may thus have received meat, eggs or whole grain cereals in the hospital diet.

Moreover, if such substances were present in the diet used by Greenspon, the administration of the gastric juice to the fasting patient only four hours before the succeeding meal is not a precaution necessarily adequate to prevent effective contact between gastric juice and food (extrinsic) factor. This is shown by the observations on patient 70. This patient received each morning 200 Gm. of beef muscle. Six hours later he was given 100 cc. of gastric juice collected as usual in our experiments and neutralized only immediately before administration. As will be seen from the data presented in table 1, a reticulocyte peak of 9 per cent resulted on the twelfth day of this regimen. The initial level of the red blood cells was 1.57, and on the fourteenth day the count reached 2.45 million per cubic millimeter. Clinical improvement was obvious. It is therefore clear that effective interaction can occur between extrinsic and intrinsic factors given six hours apart and so may have occurred in the four hour separation of the gastric juice from the next meal employed by Greenspon.

Furthermore, since the gastric juice used in this particular experiment produced positive responses in conjunction with beef muscle, it is clear that the special precautions advocated by Greenspon to prevent peptic activity are not essential to the preservation of the intrinsic factor. Additional evidence for this is obtained in the positive responses occurring with mixtures of gastric juice and extrinsic factor in the final periods in cases 62, 63, 65, 66, 68 and 69 and in the first period in case 67 (table 1). In all these instances gastric juice was collected as in our former observations, with no attempt to maintain its neutrality and so to prevent peptic activity.

The activity of desiccated hog stomach mucosa is due to the presence of both intrinsic and extrinsic factors.

We[1c] have observed in a few instances that positive effects on blood formation in pernicious anemia were obtained from the daily administration of as little as 30 Gm. of fresh hog stomach mucosa. Greenspon[11] states that "since Castle's theory is founded on the belief that the addition of beef or some 'other source of extrinsic factor' is necessary for the production of the antipernicious anemia principle, these positive results with gastric mucosa alone require explanation." Now, since the muocsa is obviously not entirely composed of gastric juice, it is clear that something besides intrinsic factor is present in it. If that something else were a source of extrinsic factor, the possibility of an effective interaction occurring could not be excluded. Greenspon's argument that, since gastric mucosa alone is effective, normal human gastric juice alone must also be effective is therefore not logical.

The following observation demonstrates that by a procedure known to destroy intrinsic factor in normal human gastric juice, hog stomach mucosa is rendered inert. To patient 7a was given daily 200 Gm. of hog stomach mucosa which had been incubated in the

13. The observation on Patient 64 was conducted by Dr. C. P. Rhoads of the Rockefeller Hospital, who has kindly allowed us to include his results.

14. Flood, Charles, and West, Randolph: Some Properties of Castle's Intrinsic Factor, Proc. Soc. Exper. Biol. & Med. 34:542 (May) 1936.

15. Hanes, F. M.; Hansen-Prüss, O. C., and Edwards, J. W.: The Feeding of Modified Gastric Juice in Pernicious Anemia, J. A. M. A. 106:2058 (June 13) 1936.

16. Ungley, C. C., and Moffett, Robert: Observations on Castle's Intrinsic Factor in Pernicious Anaemia, Lancet 1:1232 (May 30) 1936.

16a. Fitz-Hugh, Thomas, Jr., and Creskoff, A. J.: Experiments with "Depepsinized" Human Gastric Juice in the Treatment of Pernicious Anemia, Am. J. M. Sc. 192:168 (Aug.) 1936.

17. Castle, W. B.: The Etiology of Pernicious Anemia and Related Macrocytic Anemias, Ann. Int. Med. 7:2 (July) 1933.

presence of hydrochloric acid and native pepsin at pH 2.5 to 3.5 for at least forty-eight hours at 37.5 C. No evidence of increased blood formation was observed during a first period of twelve days, although the patient subsequently responded in a second period to the daily oral administration of liver extract-Lilly (N. N. R.) derived from 600 Gm. of liver (table 1).

The following observations show that extrinsic factor is present in hog stomach mucosa. About 5 Kg. of hog stomach was boiled for two hours on a water bath, then cooled, finely minced, and together with the liquor obtained, 2,500 cc. of water, 90 Gm. of pepsin, and sufficient concentrated hydrochloric acid to maintain an acidity of less than pH 2.5, was incubated at 37.5 C. for seventy-two hours. At the end of that time the liquefied material was concentrated by vacuum distillation until 100 Gm. was equivalent to 200 Gm. of original mucosa. In this process there were thus employed two procedures known to destroy the activity of hog stomach mucosa;[1c] first, boiling for at least five minutes and, second, digestion with pepsin and hydrochloric acid for forty-eight hours at 37.5 C.

To patient 66 in the second period and to patient 67 in the first period were given daily 100 Gm. of this inactivated hog stomach mucosa concentrate and 150 cc. of gastric juice immediately after admixture and neutralization with concentrated sodium hydroxide. As will be seen in table 1, patient 66 responded moderately with a reticulocyte peak of 7.5 per cent on the tenth day of the regimen. At this time the blood nonprotein nitrogen, which had been slightly elevated throughout, reached 73.8 mg. per hundred cubic centimeters, and the observation was discontinued. Patient 67 responded to a similar regimen with a reticulocyte peak of 35.9 per cent on the twelfth day. From an initial level of 0.82 million the red blood cells increased to 1.85 million per cubic millimeter on the twentieth day. Clinical improvement was correspondingly striking. Hog stomach mucosa therefore contains extrinsic factor and the probable basis for the activity of this material or, as suggested before, of whole hog stomach,[18] depends on the presence of both a thermostable (extrinsic) factor and a thermolabile (intrinsic) factor.

Incubation of normal human gastric juice for two hours at 37.5 C. inactivates only a portion of the intrinsic factor.

Greenspon[11] states that the well known hematopoietic activity of ventriculin (desiccated hog stomach) is completely destroyed in the presence of pepsin and hydrochloric acid by incubation at 38 C. for two hours or longer. The reaction of the incubated mixture was acid to congo red. From this observation he infers that in our experiments in which acid and pepsin containing gastric juice was incubated for two hours at 37.5 C. its "antipernicious anemia principle" was similarly destroyed. He presents, however, no direct evidence for this conclusion from observations with incubated gastric juice.

That the incubation of normal human gastric juice under the conditions of former observations[1b] destroys only a portion of its content of intrinsic factor is shown by the positive effects on blood formation in the second period in case 63 and the first period in case 65 (table 1). Two hundred and fifty cubic centimeters of normal human gastric juice containing active pepsin, as shown by Mett's tubes, and having a natural pH of about 1.5, was incubated for two hours at 37.5 C. Immediately thereafter the gastric juice was neutralized and given daily to each patient coincidentally with a meal containing 200 Gm. of beef muscle. In case 62 the gastric juice was incubated for only one hour and a similar positive result was observed in the second period.

Since we[19] had previously shown, however, that the incubation of normal human gastric juice for three days at 40 C. completely abolished its content of intrinsic factor, it seemed very likely that some destruction of this component would be produced by incubation for two hours at 37.5 C. Accordingly, in the third period in case 63 and in the second period in case 65 the conditions of the preceding period were exactly reproduced except that the gastric juice was not incubated but was given each day immediately after neutralization and coincidentally with a meal containing 200 Gm. of beef muscle. In the third period in case 63 there was a second reticulocyte response, reaching 14.5 per cent on the tenth day, and in case 65 a second peak of reticulocytes of 15.9 per cent was attained on the tenth day of this regimen. The occurrence of such second rises of reticulocytes indicates that the material given in these periods was more effective than that given in the preceding periods.[12] Greenspon's belief in the destructive action of peptic hydrolysis on an "antipernicious anemia principle" is thus sustained in that a two hour period of incubation is shown to be detrimental to intrinsic factor. However, since removal of pepsin without change in other properties of the gastric juice was not undertaken, our observations clearly do not permit the further definition of the nature of the destructive process as necessarily peptic hydrolysis.

Beef muscle and gastric juice administered without opportunity for contact are wholly ineffective.

Since the intrinsic factor of normal human gastric juice is partially destroyed by incubation for two hours at 37.5 C. at pH 1.5, the completely negative results of the observations[1b] in the control periods in cases 13, 15 and 17 may be questioned. In these observations such incubated gastric juice was given to the patient each day sufficiently long before the beef muscle as presumably to diminish greatly any opportunity for contact between these substances within the alimentary tract.

A repetition of these observations was undertaken without preliminary incubation of the gastric juice. During the first period in case 69, 200 Gm. of beef muscle was given to the patient at 8 o'clock in the morning. Twelve hours later 150 cc. of gastric juice was neutralized and immediately given. This regimen was

18. (*a*) Castle, Townsend and Heath.[1c] (*b*) Sturgis, C. C., and Isaacs, Raphael: Treatment of Pernicious Anemia with Desiccated, Defatted Stomach, Am. J. M. Sc. **180**:597 (Nov.) 1930.

19. Castle, W. B.; Townsend, W. C., and Heath, C. W.: Further Observations on the Etiologic Relationship on Achylia Gastrica to Pernicious Anemia, J. Clin. Investigation 9:2 (Aug.) 1930.

repeated daily for twelve days without detectable effect on blood production, as shown in table 1. In the immediately succeeding second period, however, when each day similar quantities of neutral gastric juice and beef muscle were given together, a reticulocyte peak of 20.6 per cent was reached on the tenth day and the red blood cells increased from an initial level of 1.34 to 1.95 million per cubic millimeter on the sixteenth day. In order that the amount of both beef muscle and gastric juice administered in each period of twelve days might be the same, these substances were not given after the twelfth day of the second period.

To patient 68 were given on the odd numbered days of the first period 300 Gm. of beef muscle and on the even numbered days 150 cc. of neutral gastric juice. In all, six administrations of each substance were made on alternate days during the twelve days of this first period. No detectable effect on blood production was observed. During the immediately succeeding period of twelve days, 300 Gm. of beef muscle and 150 cc. of gastric juice neutralized immediately before administration were given together every other day for six such administrations. A moderate effect on blood production occurred. The reticulocytes reached a peak of 7.4 per cent on the sixteenth day and the red blood cells did not increase. This relatively slight effect on reticulocyte production is probably explained by the presence of cystitis with fever complicating the patient's condition and by the fact that since the material was administered only on alternate days the amount given was spread over twice as long a period as in observations in which daily administration was practiced.

The observations on patients 68 and 69 demonstrate that the conclusions reached on the basis of former observations[1b] on patients 13, 15 and 17 were correct; namely, if beef muscle and gastric juice are administered without opportunity for contact, they are not effective. It is obvious, therefore, that the activity of mixtures of beef muscle and gastric juice cannot be due to the simple addition of two subthreshold substances but requires an interaction between them.

Former experiments apparently demonstrating the absence of extrinsic factor from certain substances are not necessarily valid.

Observations apparently demonstrating the negative effects of gastric juice incubated with various substances were made in case 5 (cornstarch),[1a] case 19 (washed casein),[1b] case 24 (beef muscle protein),[1e] case 27 (wheat gluten), case 51b (animal nucleic acid), cases 52, 53 and 54 (spleen pulp), cases 58 and 59 (nucleoprotein), and cases 59a, 60 and 61 (yeast nucleic acid).[1e] Since incubation of 250 cc. of gastric juice for two hours at 37.5 C. in the presence of native pepsin and hydrochloric acid at a reaction of *p*H 1.5 detectably diminishes its content of intrinsic factor, the apparently negative results of these former observations need reconsideration.

In table 2 are summarized the amounts of gastric juice, the nature of the substrate, and the reaction and duration of the incubation period in the foregoing cases. Patient 5 was given daily the entire incubated gastric contents of a normal man removed one hour after the ingestion of a meal of 300 Gm. of cornstarch. Since the incubation period in the observation on this patient lasted six hours at a reaction of *p*H 1.5 to 2, the negative result cannot be accepted. It is probable, however, from the nature of the basal diet used in all our observations that refined carbohydrate does not contain extrinsic factor. The negative result of observations on patient 19, who was given daily 50 Gm. of washed casein (A. H. Thomas Company) incubated with 300 cc. of gastric juice from three to five hours at *p*H 2.5-3.5, likewise cannot be accepted because of the prolonged incubation period.

The negative results with spleen pulp and gastric juice in cases 53 and 54 we now believe cannot be accepted, owing to the fact that the observations were made on patients in another city to which the material had to be transported, subject to delay. In former unpublished observations with incubated mixtures of beef muscle and gastric juice known to be fully effective under usual conditions, negative results were obtained when the material was so transported. In the first periods in cases 50 and 51, and in the second period in case 58, positive results were obtained from the daily administration of 50 Gm. or more of spleen pulp or a subfraction after incubation with from 50 to 75 cc. of gastric juice for two hours at p_H 7. Therefore, the negative result in patient 52, who received 100 Gm. of spleen pulp incubated for two hours with 75 cc. of gastric juice at p_H 7, seems to be clear cut. It is thus probable that, as was formerly stated, the divergent results of these observations with spleen pulp are due to variations in the content of extrinsic factor of the spleen.

In cases 24, 27, 52, 58, 59a and 60, the incubation period did not exceed two hours. Assuming the correctness of Greenspon's belief that peptic activity is responsible for the inactivation of gastric juice, the conditions (table 2) of none of these incubation procedures could have been as favorable for destruction of the intrinsic factor as those obtaining during incubation of gastric juice alone for two hours. In the latter case the reaction (p_H 1.5) was almost optimal for peptic hydrolysis and there was no substrate present potentially capable of adsorbing pepsin and so affording protection for the intrinsic factor.

In the observations in cases 52, 59, 59a and 60 in which only 75 cc. of gastric juice was employed, the reaction of the incubated mixture was p_H 6 to 7. In cases 51b and 61 only 50 cc. of gastric juice was incubated for four hours at p_H 7 with the substrates. It is possible that, although incubation of 250 cc. of gastric juice for two hours under optimal conditions for peptic activity only partially destroyed its content of intrinsic factor, the inactivation of a smaller quantity of gastric juice would be sufficiently great to produce the negative results observed. It is also possible that the temperature alone and not the peptic hydrolysis suggested by Greenspon is responsible for the partial inactivation of the intrinsic factor observed. Helmer, Fouts and Zerfas,[4] however, have obtained moderately positive effects from the daily administration of as little as 10 and 25 cc. respectively of gastric juice incubated for four hours at 47 C. with liver extract-Lilly (N. N. R.) derived from 100 Gm. of liver. The daily administration of such an amount of that liver extract alone is essentially ineffective. Because of these facts, it

does not seem probable that the reduction of the amounts of gastric juice employed in some of these observations or the incubation in some instances for as long as four hours at p_H 7 could have been responsible for the negative results. Nevertheless, because of the variability of response to oral administration among patients with pernicious anemia, negative results, unless obtained under optimal conditions for interaction between intrinsic and extrinsic factors, cannot be accepted as confidently as positive responses. For this reason the negative observations with washed casein and with certain other substances are being repeated without preliminary incubation.

Unfortunately, criticism may also be justified in respect to the negative results of others who have likewise incubated certain substances for over two hours with acid gastric juice. Thus Diehl and Kühnau[20] and Groen[2] incubated lactoflavin[20a] for three and four hours respectively with gastric juice and obtained no effect on blood production in pernicious anemia. The negative result obtained by Wills and Naish[21] with an extract of egg white incubated with gastric juice for two hours, and confirmed by Groen[2] with egg white after a four hour incubation period, contrasts with the positive result reported by Miller and Rhoads[6] after an incubation period of only one hour. On the other hand, those substances giving negative results in patients with tropical macrocytic anemia may safely be accepted as lacking in extrinsic factor provided the positive effects with other substances are due to the natural presence of intrinsic factor in the gastric juice of these patients.[22] Thus, Wills[23] found that dried yeast, a watery extract of yeast, a vitamin B_2 preparation derived from egg white, and a preparation of vitamins B_1 and B_4 adsorbed on acid clay, in contrast to various preparations of autolyzed yeast (marmite), had no blood-forming activity in tropical macrocytic anemia. In confirmation of this the Lassens[24] found that pressed top yeast and watery extracts of such yeast before or after autoclaving for one hour at 2.5 atmospheres did not lead to increased blood production in pernicious anemia after incubation with gastric juice for two hours.

COMMENT

When it is shown that for the secretion of the normal stomach to be effective in pernicious anemia a food factor is essential, the demonstration that a disturbance of the stomach is a primary factor in the immediate causation of the disease becomes possible on purely experimental grounds. Whether the fact of failure of the secretion of the stomach in pernicious anemia were known or not, a repetition of the observations which we have conducted with beef muscle and gastric juice would, we believe, lead to the conclusions that we have reached.

Briefly, these experiments have shown that in pernicious anemia the oral administration of either beef muscle or gastric juice alone is without effect. The oral administration of the gastric contents of a normal subject removed after the ingestion of a meal of beef muscle[1a] or the oral administration of mixtures of gastric juice with beef muscle,[1b] eggs,[8] autolyzed yeast,[37] wheat germ,[1e] rice polishings[38] or tomato extract[2] has been shown to produce increased blood formation in pernicious anemia. A similar process would clearly take place in the normal subject in the natural course of the digestion of certain foods.

TABLE 2.—*Conditions During the Incubation at 37.5 C. of Mixtures of Normal Human Gastric Juice with Various Substrates Administered with Negative Results to Patients with Pernicious Anemia*

Case Number and Reference of Previous Report	Gastric Juice, Cc.	Substrate		Incubation Period	
		Nature	Amount, Gm.	Duration, Hours	Reaction, p_H
5 [1a]	200	Cornstarch	300	6	1.5-2.0
19 [1b]	300	Washed casein	50	3-5	2.5-3.5
24 [1c]	150	Beef muscle protein	*	2	2.0
27 [1c]	150	Wheat gluten flour	100	2	3.0
52 [1c]	75	Spleen pulp	100	2	6.0-7.0
53 [1c]	50	Spleen pulp	50	0	7.0
54 [1c]	50	Spleen pulp	50	0	7.0
58 [1c]	100	Nucleoprotein	5	2	3.5-4.5
59 [1c]	75	Nucleoprotein	5	2	6.0
51b [1c]	50	Animal nucleic acid	5	4	7.0
59a [1c]	75	Yeast nucleic acid	10	2	6.0
60 [1c]	75	Yeast nucleic acid	10	2	6.5
61 [1c]	50	Yeast nucleic acid	5	4	7.0

*Derived from 200 Gm. of beef muscle.

Our conception of the experimentally demonstrable factors normally involved in the production of the substance that is deficient in the liver of patients with pernicious[35] and related macrocytic anemias[36] is represented by the schematic formula

$$\frac{F \times G}{I} = L.\ E.$$

Here F stands for food (extrinsic) factor, G for gastric (intrinsic) factor and I for intestinal impermeability or any defect causing malabsorption or destruction of those substances or a product of their effective interaction. *L. E.* stands for "liver extract," the independently effective thermostable factor found in mammalian liver, kidney and certain other organs. Probably in none of the anemias referred to is any factor on the left of the equation completely normal, and in every instance there

20. Diehl, F., and Kühnau, J.: Ist Vitamin B_2 der therapeutisch wirksame äussere Faktor beim Morbus Biermer? Deutsches Arch. f. klin. Med. **176**:149 (Dec. 12) 1933.

20a. We have, however, now entirely confirmed these negative results by giving a mixture of 150 cc. of neutralized gastric juice and 20 mg. of lactoflavin daily for five days without preliminary incubation. We are indebted to Vitab Products, Inc., for supplying the lactoflavin.

21. Wills, Lucy, and Naish, Alice: A Case of Pernicious Anaemia Treated with Vitamin B_2 from Egg White, Lancet **1**:1286 (June 17) 1933.

22. Strauss and Castle.[1c] Ungley and James.[9]

23. Wills, Lucy: Studies in Pernicious Anaemia of Pregnancy: VI. Tropical Macrocytic Anaemia as a Deficiency Disease, with Special Reference to the Vitamin B Complex, Indian J. M. Research **21**:669 (April) 1934.

24. Lassen, H. C. A., and Lassen, H. K.: Yeast or Vitamin B_2 as "Extrinsic Factor" in Treatment of Pernicious Anemia, Am. J. M. Sc. **188**:461 (Oct.) 1934.

37. Strauss and Castle.[1c] Groen.[2]

38. Strauss and Castle.[1c] Miller and Rhoads.[6]

is a variable participation of defects of one or both of the factors in the numerator[39] or some increase of the denominator value.[40] Any or all such changes from the normal will, however, result in a decrease of "liver extract" which, if sufficiently great, may allow the development of a macrocytic anemia which will respond to the parenteral administration of liver extract derived from the liver of a normal animal.

If the dominant defect is of food (extrinsic) factor, the anemia will respond both to orally administered extrinsic factor and to liver extract (e. g., macrocytic anemia of pregnancy in the tropics[41] and elsewhere[39b] or of certain cases of sprue[36] and idiopathic steatorrhea[42]). The presence of some intrinsic factor in the stomach probably explains the occurrence of "spontaneous" remissions in certain cases of pernicious anemia[43] as well as the usual recovery following delivery of patients with the pernicious anemia of pregnancy.[39b] Likewise the partial success of former methods of treatment with high protein diets in both pernicious anemia[1d] and sprue[36] was probably due to a similar effect. On the other hand, the concept of a defect of an independently active antipernicious anemia principle secreted by the stomach, as proposed by Morris[25] and by Greenspon,[11] does not satisfactorily explain the immediate etiologic mechanism of those instances of macrocytic anemia in which intrinsic factor is demonstrably present in the gastric contents.[36] If the dominant defect is of gastric (intrinsic) factor, the anemia will not respond to orally administered extrinsic factor unless gastric juice is given simultaneously but will respond to liver extract administered orally or parenterally (e. g., addisonian pernicious anemia).

The existence of an essential preliminary reaction between food and gastric juice does not, however, preclude the possibility of defects of other subsequent and essential reactions within or without the alimentary tract. If intestinal impermeability is sufficiently increased, the patient will not respond normally to mixtures of extrinsic and intrinsic factor or to stomach or liver preparations given by mouth but will respond to parenterally administered liver extract (e. g., macrocytic anemia of chronic sprue[36] or of intestinal stenoses or short circuits[44]). In theory at least, failure or inhibition of any essential link in the further metabolism within the body will likewise diminish the supply of liver extract available to the bone marrow.[45] It is certainly clear that infections[12] may have an inhibitory effect on the action of liver extract in pernicious anemia. This concept of the etiologic relationships between pernicious anemia and other types of macrocytic anemia which likewise respond to the parenteral administration of liver extract has been fully discussed elsewhere.[46]

CONCLUSIONS

The following observations on patients with pernicious anemia fail to sustain the conclusions of Greenspon:

1. Normal human gastric juice does not contain, on oral administration, an "antipernicious anemia principle" effective without contact with food (extrinsic) factor.
2. Hog stomach mucosa contains both a thermostable (extrinsic) factor and a thermolabile (intrinsic) factor presumably responsible for the activity of such mucosa and of whole desiccated hog stomach.
3. Incubation of normal human gastric juice for two hours at 37.5 C. in the presence of native pepsin and hydrochloric acid inactivates only a portion of its content of intrinsic factor.
4. Beef muscle (extrinsic factor) and gastric juice (intrinsic factor) administered without opportunity for contact are not effective in pernicious anemia.

Greenspon's recent experiments have led to the following modified conclusions in respect to former observations:

1. The negative results of the administration of substances after incubation with gastric juice for longer periods than two hours at 37.5 C. at an acid reaction cannot be accepted.
2. Lack of extrinsic factor in substances so incubated with gastric juice is not established by negative results.
3. Preliminary incubations should not be employed in testing the blood-forming activity in pernicious anemia of mixtures of gastric juice and various substrates.

39. (*a*) Castle, W. B.: The Etiology of Pernicious and Related Macrocytic Anemias, Science **82**:159 (Aug. 23) 1935. (*b*) Strauss, M. B., and Castle, W. B.: Studies of Anemia in Pregnancy: III. The Etiologic Relationship of Gastric Secretory Defects and Dietary Deficiency to the Hypochromic and Macrocytic (Pernicious) Anemias of Pregnancy and the Treatment of These Conditions. Am. J. M. Sc. **185**:539 (April) 1933. (*c*) Goldhamer, S. M.: The Presence of the Intrinsic Factor of Castle in the Gastric Juice of Patients with Pernicious Anemia, Am. J. M. Sc. **191**:405 (March) 1936.

40. Castle, Heath and Strauss.[1d] Castle, Rhoads, Lawson and Payne.[36]

41. Wills:[23] Treatment of "Pernicious Anaemia of Pregnancy" and "Tropical Anaemia," with Special Reference to Yeast Extract as a Curative Agent. Brit. M. J. **1**:1059 (June 20) 1931.

42. Vaughan, J. M., and Hunter, Donald: The Treatment by Marmite of Megalocytic Hyperchromic Anaemia Occurring in Idiopathic Steatorrhoea (Coeliac Disease), Lancet **1**:829 (April 16) 1933.

43. Castle, Heath and Strauss.[1d] Goldhamer.[39c]

44. Castle, Heath and Strauss.[1d] Schlesinger, Annemarie: Nachweis des Antiperniciosa-Prinzips im Magensaft einer Patientin mit perniziösanämischem Blutbild bei Dünndarmstenose, Klin. Wchnschr. **12**:298 (Feb. 25) 1933. Strauss, M. B.: The Role of the Gastro-Intestinal Tract in Conditioning Deficiency Disease: The Significance of Digestion and Absorption in Pernicious Anemia, Pellagra and "Alcoholic" and Other Forms of Polyneuritis, J. A. M. A. **103**:1 (July 7) 1934.

45. Castle, Heath and Strauss.[1d] Wintrobe, M. M., and Schumacker, H. S. Jr.: The Occurrence of Macrocytic Anemia in Association with Disorder of the Liver. Bull. Johns Hopkins Hosp. **52**:387 (June) 1933. Goldhamer. S. M.: Liver Extract Therapy in Cirrhosis of the Liver: Relation of Liver Dysfunction to Nonstorage of "Antianemic" Substance in Producing a Blood Picture Resembling Pernicious Anemia in a Patient Secreting Free Hydrochloric Acid, Arch. Int. Med. **53**:54 (Jan.) 1934.

46. Castle, Heath and Strauss.[1d] Strauss and Castle.[1e] Castle.[17] Castle.[39a] Strauss and Castle.[39b]