Enhanced Absorption of Vitamin B_{12} in Gastrectomized Rat by Rat Intrinsic Factor.* (22253)

H. O. Nieweg,† A. Arends, E. Mandema, and W. B. Castle.

Thorndike Memorial Laboratory and Second and Fourth (Harvard) Medical Services, Boston City Hospital, Department of Medicine, Harvard Medical School, and Departments of Pathology and of Medicine, University of Groningen, Holland.

The essential physiologic defect in Addisonian pernicious anemia and in the totally gastrectomized human subject is inability to assimilate vit. B_{12}. In both, this absorption defect can be diminished by the simultaneous administration of the so-called intrinsic factor of normal human gastric juice. This specific action of intrinsic factor is demonstrable following its simultaneous oral administration with vit. B_{12} in two ways: (a) hematopoietic effects in the anemic patient(1) or (b) enhanced uptake of radioactive vit. B_{12} in either the anemic or the non-anemic patient whether previously treated or not(2-4) including the gastrectomized patient(5). To date, no other method of detecting the activity of the intrinsic factor of normal human gastric juice has been found to be satisfactory.

The availability of radioactive vit. B_{12} labelled with Co^{60}(6), and especially of preparations of high specific radioactivity, suggested an attempt to develop an animal preparation for the detection of intrinsic factor. Although it was not known whether the rat, like man and the hog, secretes intrinsic factor, the availability of the species as a laboratory animal appeared to justify an effort to employ it. The fact that no increase, or indeed a decrease, in the uptake of radioactive vit. B_{12} had been found by others(7) to occur in the intact rat as a result of the simultaneous administration of intrinsic factor and vit. B_{12} was not discouraging, since this would be expected if the natural secretion of intrinsic factor was unimpaired and if non-specific binding(8) of Co^{60}-B_{12} by the intrinsic factor preparations had occurred. Our first interest was to discover whether the removal of the stomach and/or other portions of the gastrointestinal tract would reduce the ability of the rat to absorb radioactive vit. B_{12}. It was soon found, as recently reported by others(9), that with total gastrectomy this was so. However, it was considered that such a mutilating operation on the alimentary tract might well have had entirely non-specific effects on assimilation including vit. B_{12}. Consequently, only if an enhanced absorption of vit. B_{12} ensued in the operated animal when intrinsic factor was simultaneously administered by mouth could the conclusions be drawn with certainty that deficiency of intrinsic factor secretion (a) had been induced by the operative procedure and (b) had been temporarily abolished by the intrinsic factor administered. These conclusions appear to be justified by the present observations upon totally gastrectomized rats using Co^{60}-labelled radioactive vit. B_{12}, but only when normal rat stomach was the source of the intrinsic factor administered.

Methods. Male white rats of the Sprague-Dawley strain, weighing about 250 g were used. Total gastrectomy was done under Nembutal (pentobarbital sodium) anesthesia producing an end-to-end anastomosis of the lower end of the esophagus to the bisected pylorus. After brief experience the mortality at operation and during the following week was about 30%. Among animals surviving 2 weeks, only occasional animals lost weight and died. Gastrectomized rats were not used in experiments until at least 10 days after operation and only if they appeared to be in good health. At least 5 days elapsed between successive administrations of Co^{60}-B_{12} on the same gastrectomized animal. During the experiments the animals were kept in individual metabolism cages permitting total collection of stools without coprophagy. In order to

* The expenses of this investigation were defrayed in part by the J. K. Lilly gift to the Harvard Medical School and by a grant from N. V. Philips-Roxane, Weesp, Holland.

† Fellow, Netherlands Organization for Pure Research Z. W. O.

minimize the bulk of the feces of which the radioactivity was to be measured, the diet supplied *ad libitum* consisted of a nutritionally complete synthetic low residue diet.‡ It contained 18% casein as the source of protein, and among other essentials 1.35 mg vit. B_{12}/100 lb diet. On this diet the gastrectomized rats appeared to be healthy, gained weight and excreted normal appearing feces. After a preliminary fast of at least an hour each animal was lightly anesthetized with ether and given a single dose of Co^{60}-B_{12} followed immediately by the preparation to be tested for intrinsic factor activity. Both were in liquid form and were injected with different syringes in measured amounts by means of a small rubber catheter about 3 mm in diameter that had been cautiously inserted through the esophagus for a distance of 8-10 cm. The amount of radioactive vit. B_{12}§ administered, which had a specific activity of about 1 $\mu c/\mu g$, was invariably 0.015 μg dissolved in 1 ml of water. The volume of the material to be tested for intrinsic factor activity was the same in each experimental group of rats and ranged in different experimental groups from 2-5 ml for a single dose. The stools were then collected over a 4-day period, digested with 20% NaOH and homogenized in a Waring Blendor. Thereafter 4 aliquot samples of 7.5 ml each were examined for radioactivity in a well-type scintillation counter and the individual values averaged. Human gastric juice was obtained from a normal fasting male following the injection of Histalog (3-beta aminoethylpyrazole dihydrochloride)(10). The juice was neutralized immediately after collection, passed through a coarse sintered glass filter and kept frozen until used. This procedure is known from many observations on patients with pernicious anemia(1) to provide an active source of intrinsic factor. As a source of hog intrinsic factor, a commercial preparation of hog stomach mucosa‖ was used. As a potential source of "rat intrinsic factor" the entire stomachs of normal rats were homogenized in a Waring Blendor with water. For each stomach, which had been kept frozen since excision and which weighed about 1.5 g, 10 ml of water were used in preparing the homogenate which was filtered through gauze in order to remove coarse particles before use.

Rat gastric juice was obtained from normal animals fasted overnight following ligation of the pylorus. In the morning a variable degree of gastric distention was noted when the animal was killed and the gastric contents, averaging about 15 ml, were removed. The gastric juice so obtained was slightly turbid and sometimes brownish in appearance. The pH of a pooled sample from several rats was 1.3.

Results. The results of measurements of the radioactivity in the feces in single experiments upon 11 normal rats and in 61 experiments upon 14 gastrectomized rats are graphically presented in Fig. 1. Values are reported as percentage recoveries of radioactivity after single oral doses of Co^{60}-B_{12} together with water or with one of the preparations to be tested for intrinsic factor activity.

In *normal rats* a significant fraction of the test dose of Co^{60}-B_{12} in water was usually absorbed as inferred from the excretion of radioactivity. In the *gastrectomized rats* there was apparently no absorption of Co^{60}-B_{12}. When 2 ml of normal human gastric juice were used as a source of intrinsic factor, the absorption of vit. B_{12} did not improve, nor was the fecal excretion diminished when, instead of 2 ml of normal human gastric juice given once, 3 ml were given initially and were followed after an interval of an hour by 3 ml more. The administration of 4 mg and even of 20 mg of the preparation of hog stomach mucosa was also ineffective in decreasing the excretion of radioactivity.

On the other hand, when whole rat stomach homogenized in water was administered in a dosage of ½ of a stomach to gastrectomized rats the absorption of vit. B_{12} apparently became normal. When the dose was decreased to 1/10 of a stomach, the effect upon fecal

‡ Available commercially from Nutritional Biochemicals Corp., Cleveland, O.

§ Kindly supplied by Dr. Nathaniel S. Ritter of Merck & Co., Rahway, N. J.

‖ Obtained through the courtesy of Dr. Kenneth W. Thompson of Organon Inc., Orange, N. J.

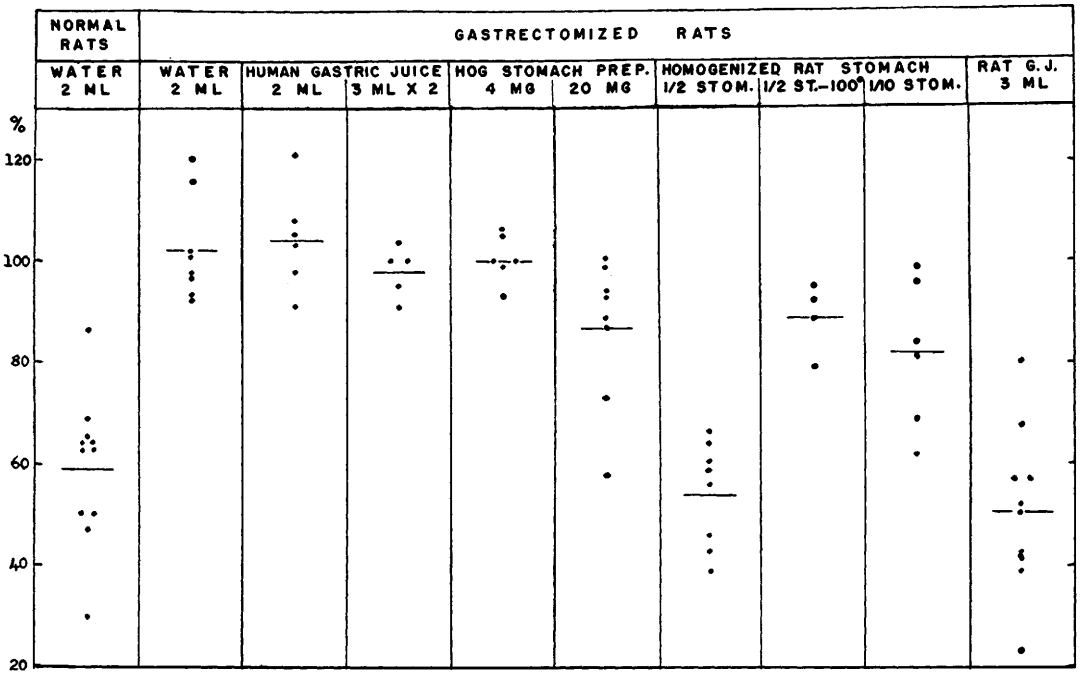

FIG. 1. Radioactivity recovered in the feces expressed as percentage of the single dose of 0.015 μg Co^{60}-B_{12} administered together with the substances indicated above. Each dot represents the result of an experiment on a single animal; bars indicate averages.

excretion was at most slight. When 3 ml of rat gastric juice were administered, the average reduction in fecal radioactivity resembled that obtained with ½ of a homogenized rat stomach. Finally, when ½ of a homogenized rat stomach was administered subsequent to heating on a boiling water bath for 7 minutes, its effectiveness appeared to be greatly diminished.

Discussion. These results appear to demonstrate that total gastrectomy in the rat renders the animal unable to absorb a small test dose of radioactive vitamin B_{12} when given in an amount of which about half is absorbed by the normal animal. This disability is not associated with any gross signs of intestinal dysfunction, and may be largely abolished by the simultaneous administration of sufficient homogenized rat stomach or rat gastric juice. Taken together, these findings indicate that the assimilation of vit. B_{12} in the rat resembles that in man in that the stomach appears to be the exclusive or major site of secretion of a so-called intrinsic factor essential for that process. However, neither normal human gastric juice nor the preparation of hog stomach mucosa employed displayed with certainty any activity as intrinsic factor in the rat.

During the preparation of this manuscript our attention was drawn to the recent publication of Watson and Florey(11) demonstrating that after resection merely of the distal (secretory) portion of the rat stomach results were obtained that were generally similar to ours. Thus, their operated animals failed to absorb radioactive vit. B_{12}, and this difference from the intact animal was corrected by administration of extracts prepared from the distal portion of the rat stomach but not by administration of the secretion from a pyloric pouch created in the hog. It is a pleasure to report this confirmation of their observations and to point to their additional evidence that in the rat the secretion of intrinsic factor is largely by the distal portion of the stomach. Moreover, although not established by the experiments of Chow and his associates(9), it now appears probable that the defective absorption of Co^{60}-B_{12}

reported by them was in fact due to lack of rat intrinsic factor.

The apparent inactivity of normal human gastric juice and of hog stomach preparations employed by others(9,11) as well as here requires consideration. In our experiments both were obtained by methods repeatedly shown to conserve their intrinsic factor activity for patients with pernicious anemia. The doses of human gastric juice employed, 3 and 6 ml respectively, were for the rat relatively much larger than the 10 ml known to be hematopoietically active with as little as 1 μg of vit. B_{12} in pernicious anemia(12). The administration of 4 mg of the hog stomach preparation was without detectable effect as was also in all probability the administration of 20 mg. The larger amount constitutes about ½ of the amount of hog stomach extract present in 1 U.S.P. unit of a pharmaceutical preparation of vit. B_{12} with Intrinsic Factor Concentrate.** This is an effective daily oral dose in pernicious anemia when given with about 15 μg of vit. B_{12}. This hog stomach preparation contained about 80 $\mu\mu$g of normal vit. B_{12} in the 20 mg dose or far less than the 1680 $\mu\mu$g present in ½ of a homogenized rat stomach. Moreover, 6 ml of normal human gastric juice is said to contain only about 0.004 μg of vit. B_{12}(13). Thus, in these preparations insufficient normal vit. B_{12} was administered to compete significantly for absorption with the Co^{60}-B_{12} and so to account for their apparent inactivity in the rat. Although the inhibitory effect of non-specific binding(7,8) of the vit. B_{12} by the human and hog intrinsic factor preparations can not be excluded, the negative results with these preparations are consistent with the speculation of Chow and his associates (9) that intrinsic factor may possess species specificity. In this connection it is of interest to note that Toporek and his associates(14) found that, in contrast to the plateau effect of hog stomach preparations, progressively more Co^{60}-B_{12} was absorbed in pernicious anemia patients when normal human gastric juice was used as a source of intrinsic factor.

Conclusions. 1. The percentage excretion of radioactivity in the feces of normal and of totally gastrectomized rats, following administration of a single dose of 0.015 μg Co^{60}-B_{12}, was determined by scintillation counting. 2. The gastrectomized rats apparently were unable to absorb the labelled vit. B_{12} (100% fecal excretion). 3. Simultaneous administration of homogenized rat stomach or of rat gastric juice but *not* of human gastric juice or of an extract of hog stomach mucosa reproduced in the gastrectomized rat the capacity of the normal rat to absorb the test dose of Co^{60}-B_{12} administered (60% fecal excretion). 4. The rat, like man, secretes intrinsic factor largely, if not exclusively, into the stomach; but sources of human and hog intrinsic factor possess little or no activity in the rat. 5. The observations of Watson and Florey indicate that in the rat the secretion of intrinsic factor is probably confined to the distal secretory portion of the stomach.

The authors are grateful to Mrs. Rose B. Blumenthal and to Mrs. Ellen B. Block of the Thorndike Memorial Laboratory for technical assistance.

¶ Subsequently confirmed here in experiments upon 6 totally gastrectomized rats given ½ of the distal portion of a rat stomach after homogenization in water. The fecal excretions ranged from 54-72, average 60%, of the radioactivity administered. On the other hand, 5 similar experiments with the proximal portion of the rat stomach showed no effect upon fecal excretion: range 99-121, average 112%.

**Information kindly supplied by Dr. Kenneth W. Thompson of Organon Inc., Orange, N. J.

1. Berk, L., Castle, W. B., Welch, A. D., Heinle, R. W., Anker, R., and Epstein, M., *New England J. Med.,* 1948, v239, 911.

2. Heinle, R. W., Welch, A. D., Scharf, V., Meacham, G. C., and Prusoff, W. H., *Tr. A. Am. Phys.,* 1952, v65, 214.

3. Schilling, R. F., *J. Lab. and Clin. Med.,* 1953, v42, 860.

4. Glass, G. B. J., Boyd, L. J., Gellin, G. A., and Stephanson, L., *Arch. Biochem.,* 1954, v51, 251.

5. Swendseid, M. E., Halsted, J. A., and Libby, R. L., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 226.

6. Chaiet, L., Rosenblum, C., and Woodbury, D. T., *Science,* 1950, v111, 601.

7. Rosenblum, C., Woodbury, D. T., Gilfillan, E. W., and Emerson, G. A., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 268.

8. Prusoff, W. H., Welch, A. D., Heinle, R. W.,

and Meacham, G. C., *Blood,* 1953, v8, 491.

9. Chow, B. F., Quattlebaum, J. K., Jr., and Rosenblum, C., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 279.

10. Rosiere, C. E., and Grossman, M. I., *Science,* 1951, v113, 651.

11. Watson, G. M., and Florey, H. W., *Brit. J. Exp. Path.,* 1955, v36, 479.

12. Wallerstein, R. O., Harris, J. W., Schilling, R. F., and Castle, W. B., *J. Lab. and Clin. Med.,* 1953, v41, 363.

13. Callender, S. T., and Lajtha, L. G., *Blood,* 1951, v6, 1234.

14. Toporek, M., Bishop, R. C., Nelson, N. A., and Bethell, F. H., *J. Lab. and Clin. Med.,* 1955, v46, 665.

Received January 20, 1956. P.S.E.B.M., 1956, v91.

Enzymatic Degradation of Side-Chain of Adrenocortical Steroids: Conversion of Hydrocortisone to Reichstein's E.* (22254)

E. MYLES GLENN,† PATRICK M. L. SIU, AND RICHARD O. RECKNAGEL.
(Introduced by George Sayers.)

Department of Physiology, Western Reserve University School of Medicine, Cleveland, O.

In recent publications from this laboratory (1,2) an enzyme system found in both microsome and supernatant fractions of rat liver has been described which induces an alteration in the C-20 ketone group of adrenocortical steroids. Further observations on this system have been made and in addition positive evidence has been obtained that the reaction involving the C-20 ketone of the steroid molecule is reductive in nature.

Methods. Methods used in this study were identical with those described in previous publications(1,2).

Results. The first study in this series was undertaken to determine if the system reducing ring A could be separated from the system reducing the 20-ketone. A 0.25 *M* sucrose homogenate of rat liver free of nuclei and mitochondria was centrifuged at 20,000 X gravity for one hour. The supernatant fraction was discarded and the microsome fraction was resuspended and recentrifuged twice under the same conditions. Results obtained following incubation (37°C, 1 hr) of cortisone with the twice-washed microsome fraction are indicated in Fig. 1. It may be noted that the extent of Δ^4-3-ketone reduction in the complete system was only slightly less than that of 20-ketone reduction. Subsequent studies have confirmed this observation.‡ These results are at variance with those of Tomkins and Isselbacher(3) who reported that the Δ^4-3 ketone reducing system is present only in the supernatant fraction of rat liver.

p-Chloromercuribenzoic acid (PCMBA) inhibition of the Δ^4-3 ketone reducing system and of the 20-ketone reducing system in the microsome fraction was investigated. Fig. 2

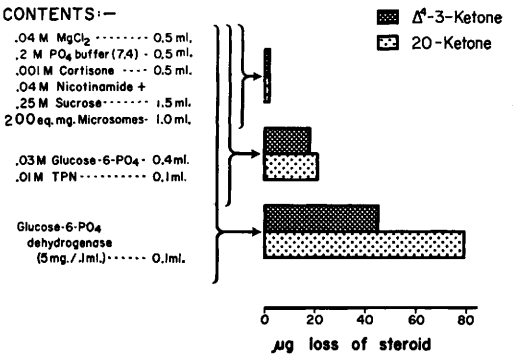

FIG. 1. Presence of both C-20 ketone and Δ^4-3-ketone reducing systems in microsome fraction of rat liver. See footnote (§) for definition of equivalent milligram (eq mg).

* This investigation was supported by research grants A-329 and A-331 from National Institute of Arthritic and Metabolic Diseases of National Institutes of Health and the Arthritis and Rheumatism Foundation.

† Data submitted in this report are in partial fulfillment for the Ph.D. degree. Predoctoral Fellow of the Arthritis and Rheumatism Foundation, 1955-1956. Present address: Upjohn Co., Kalamazoo, Mich.

‡ Recknagel, R. O., unpublished.