THE ABSORPTION OF VITAMIN B_{12} IN GASTRECTOMISED RATS.

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INVESTIGATION of Castle's intrinsic factor has long been handicapped by the lack of any means of detecting its presence other than its therapeutic effect when given, together with a source of extrinsic factor, to patients with pernicious anaemia in relapse. The recent introduction of radioactive vitamin B_{12} has permitted more extensive clinical study, for the absorption of this substance can be measured in a patient with pernicious anaemia in remission, or in a normal subject. Difficulties remain, for not all patients can be used for these studies and only a limited amount of the isotope concerned, ^{60}Co, may be given to any one subject; in consequence an animal preparation in which the effects of intrinsic factor could be examined would prove of considerable value.

In man gastrectomy causes loss of the ability to absorb orally administered vitamin B_{12}, a loss which can be made good by feeding normal gastric juice together with the vitamin (Swendseid, Halsted and Libby, 1953). It was thought that gastrectomy in animals might have a similar result, and some experiments have been done to test this possibility. Unfortunately little is known about the secretion of intrinsic factor in any animal except the pig; the gastric juice of pigs contains intrinsic factor (Landboe-Christensen and Wandall, 1953), and in gastrectomised pigs the liver loses its content of haemopoietic principle (Geiger, Goodman and Claiborn, 1937). The pig might prove a suitable animal but the practical difficulties of maintenance and the collection of faeces would be considerable. The rat was chosen for our experiments; its faeces can be collected completely in metabolism cages, and it is simple to administer vitamin or intrinsic factor preparations by a stomach tube.

EXPERIMENTAL.

Male albino rats 3–4 months old and weighing 200–220 g. were used, some being set aside as intact controls. All rats were given 7 mg. of novarsenobenzol subcutaneously to cure Bartonella infection and were subsequently kept in isolation. They were prepared for operation by fasting overnight.

Gastrectomy.

The rat stomach is divided into two well defined parts, the glandular lower half extending from the pylorus to a line which, when the stomach is empty, runs from a point on the lesser curvature 2–3 mm. from the oesophagus to about the centre of the greater curvature, and a non-glandular upper half lined with squamous epithelium. It was thought unnecessary to resect the squamous-lined part of the stomach, and the glandular part only was removed, together with approximately the first centimetre of the duodenum, which contains the main mass of Brunner's glands.

Under ether anaesthesia and with full aseptic precautions, except that gloves were not

worn, the upper abdomen was opened by a midline incision and an eyelid retractor inserted. The stomach was brought out and the vessels supplying the greater curvature from the junction of the glandular and non-glandular parts to the pylorus were ligated with fine nylon thread. Vessels supplying the pylorus and duodenum were ligated next, and then the large artery on the anterior surface of the body. As far as possible the branches to the oesophagus and non-glandular part of the stomach were preserved. Finally the stomach was turned upwards and the large vessel supplying the posterior surface was ligated as high up as possible. The omentum was then divided along the greater curvature, the duodenum cut across about 1 cm. below the pylorus, and the pylorus and duodenum cut free. After the peritoneal cavity had been packed off, the stomach was opened on the greater curvature at the junction of the glandular and non-glandular parts, and the incision was continued with scissors along the well defined glandular-squamous boundary. On the lesser curvature, where the mucosa reaches a point about 3 mm. from the oesophagus, the squamous part was carefully preserved so that the remnant of the stomach could be closed without occluding the oesophagus. For closure and anastomosis fine unbraided silk was the most suitable suture material. This was mounted on a No. 6 curved round-bodied needle as used in surgery of the eye. The stomach remnant was closed with a continuous inverting suture. Closure was begun at the top of the stomach remnant and terminated when the remaining opening was equal in size to the cut end of the duodenum. During the early stages of closure a glass rod was inserted into the oesophagus from the stomach to ensure that the opening was left patent. The distal opening in the stomach was anastomosed directly to the duodenum, using a continuous suture which inverted the cut edges. The abdomen was closed in two layers with continuous silk sutures.

Condition of rats after gastrectomy.

After operation the rats were given 5 ml. of saline and 50,000 units of penicillin subcutaneously, this dose of penicillin being given daily for a week. For the first three days after operation the rats were given only glucose water by mouth, and then gradually reintroduced to solid food. Deaths after operation were mostly from peritonitis, obstruction or haemorrhage, but in some cases an apparently healthy animal developed extreme dilatation of the remnant of the stomach and died on the first or second day after operation.

No detailed study of the blood was made in these experiments. Animals with normal haemoglobin values were selected for operation, and haemoglobin readings and blood films were made at intervals after operation. Survivors tended to develop hypochromic anaemia, and those animals which had lost much blood at operation failed to regain normal levels of haemoglobin. The anaemia was resistant to iron therapy, both by mouth and parenterally, as has been reported by Balfour, Higgins and Woods (1950).

Those rats which survived operation usually looked healthy and gained weight almost normally for several months, but they then began to decline and died in the sixth or seventh month after operation. In many cases the symptoms were those of obstruction—failure to eat, loss of weight, dehydration and regurgitation of ingested food—and it was sometimes possible to relieve these symptoms temporarily by passing a fine rubber catheter into the remnant of the stomach in order to wash it out with saline, and by giving saline subcutaneously.

Findings post mortem.

Only one rat was killed when still apparently in good health, 130 days after operation, and its tissues were fixed immediately. Microscopically the squamous epithelium of this animal's stomach was very hyperplastic and was covered by a thick layer of keratin. Here and there in the folds of keratin were masses of bacteria, and in some places there were pockets of polymorphs between the

keratin and the epithelium. The submucosa showed intense chronic inflammation and in places giant cells, which may have been associated with the stitches put into the stomach. Two ulcers penetrating to the sub-epithelial tissue were present at the oesophageal end of the stomach. To one side of one of the ulcers a remnant of a stitch could be seen, and near the base of the other were two giant cell systems, but it seems improbable, in view of the wide-spread inflammation, that these ulcers were due to the presence of unabsorbed stitches. The oesophagus showed no evidence of hyperkeratosis, but near the junction between oesophagus and stomach the epithelium gradually thickened and passed over into the greatly hypertrophic epithelium of the stomach. The junction of stomach and intestine had healed well. In the intestine the villi were well preserved and, in contrast to the state of the stomach, there was no sign of inflammation. There was a small remnant of Brunner's glands in the intestine close to the suture line.

Other animals examined post mortem died after overt illness of varying duration, and autopsies were not made immediately after death. Two of these animals showed macroscopically a large lumen at the lower end of the oesophagus.

Five rats had ulcers in the squamous epithelium of the stomach and those examined histologically showed evidence of epithelial hyperplasia and increased production of keratin, although this was not present in the oesophagus. The ulcers were associated with acute inflammatory changes and in some stomachs there was infiltration with plasma cells and macrophages. In one animal there appeared to be a remnant of Brunner's glands.

In two rats the stomach had perforated and metallic particles coming from a gnawed cage were found outside the perforation. In another a large hair ball was present.

In view of this it is difficult, in the absence of demonstrable mechanical irritation, to draw firm conclusions about the origin of the ulcers which were present in some rats.

Measurement of absorption of vitamin B_{12}.

The absorption of vitamin B_{12} was estimated by a technique similar to that introduced by Welch and Heinle (1951) for studies in man. A measured dose of vitamin B_{12}, labelled with ^{60}Co, was given by mouth, and the amount of radioactivity subsequently excreted in the faeces was measured. When normal rats are fed vitamin B_{12} in doses of 20 μg. or more, almost the whole amount is excreted in the faeces (Lester Smith, 1952; Barbee and Johnson, 1951), but experience in man suggested that if a much smaller dose were used vitamin B_{12} absorption might be studied. Preliminary results suggested that a dose of approximately 0·005 μg. of vitamin B_{12} would be suitable in that a reasonable differential between normal and gastrectomised rats might be expected, while at the same time there would be enough radioactive matter in the faeces to permit accurate counting. This dose was used throughout these experiments and gave satisfactory results, although it may not be the optimum value for such studies. The radioactive vitamin used in these experiments had an activity of 0·6 μC/μg. At the end of the experiments the residue was tested by Dr. Lester Smith, who found that it had not lost any microbiological activity.

Although the gastrectomised rats were able to take a diet of ordinary rat cake, the following diet was given to reduce the bulk of the faeces to a convenient amount: cornflour 65 g., low vitamin content casein 28 g., brewer's yeast 5 g., and salt mixture (Hubbell, Mendel and Wakeman, 1937, but with the iron content doubled), 2 g. This diet was supplemented by milk between experiments. For 24 hr. before and after feeding vitamin B_{12} the diet was replaced by a simple paste of cornflour, except for 8 hr. immediately before and after the feeding, when only water was allowed. This was an empirical arrangement intended

to permit the greatest possible absorption of the vitamin B_{12}. After the administration of the radioactive vitamin B_{12} the rats were placed in metabolism cages and kept there until radioactivity in the faeces became negligible; 8 days were usually sufficient but some experiments were carried on for 10 days. The faeces were collected for assay of radioactivity at intervals of 48 hr., care being taken to see that any faeces adherent to the cage or collecting funnel were included in the samples. Each collection was placed in a small petri dish, dried at 37° for 24 hr., and then powdered by hand in a mortar. The amount of radioactivity in the urine was too small for measurement.

Measurements of radioactivity were made by scintillation counting, using a 5 cm. sodium iodide crystal mounted on a type 6260 photo-multiplier tube operated at 800 V. (Electrical & Musical Industries Ltd., Hayes, Middlesex). This was connected to an amplifier (AERE No. 1008B) and a scaler with discriminator bias set at 10 V. This arrangement gave a background count of about 175 per min. and, with the size of sample used, an efficiency of about 18 per cent for ^{60}Co. Standards and samples of the faeces were counted in uniform thin-walled glass bottles with an internal diameter of 2·2 cm. These bottles were centred over the crystal shield by a perspex ring. The 48-hr. samples of dried and powdered faeces did not exceed 2·5 ml. in volume; they were all made up to this volume by adding some dry food mixture to the mortar before the faeces were transferred to the counting bottles. This practice also helped to prevent particles of faeces from sticking to the mortar. Background counts were made on the empty bottles before each sample was counted. Counting times were not fixed, but were carried on long enough to keep the statistical error of counting to about 2·5 per cent of the total dose, at the 5 per cent probability level.

The quantities of vitamin B_{12} used in the present experiments were very small and a system of checks was used to minimise errors. Stock solution of vitamin B_{12} was taken up by micropipette and added to 4 ml. of water to give a concentration of 0·01 μg./ml. For each experiment doses of 0·5 ml. were taken from this solution and placed in standard counting bottles. Two further quantities of 0·5 ml. were added to 2·0 ml. of water in standard bottles, and these two bottles provided radioactive standards throughout the experiment. Before administration of the vitamin all the doses and standards were counted to check their uniformity, and after administration the empty bottles were again counted, so that any residual radioactivity might be allowed for in the subsequent calculations.

The solution under test for intrinsic factor activity (or water for control animals) was given in a volume of 2·5 or 3 ml. This volume was divided into three portions; the first was given 1 hr. before feeding the vitamin, the second was incubated with the vitamin at 37° for 1 hr. before feeding, and the third was used to wash out the bottle that had contained the vitamin and was given after feeding. Feeding was done through a polythene tube with an internal diameter of 0·5 mm. which was passed into the stomach. A 14-gauge needle and a 2 ml. syringe containing the vitamin were then attached to the polythene tube and the material injected. On a few occasions, after removal of the needle, a little fluid escaped from the polythene tube; if any vitamin was lost in this way the experiment was abandoned. Finally 0·25 ml. of air was injected and the tube withdrawn. The injection of these volumes of fluid into the stomach remnant did not appear to distress the rats.

Materials tested for intrinsic factor activity.

Rat stomach extract was prepared from the glandular parts of the stomachs of freshly killed animals. The duodenum was not included. The material obtained from each rat was ground with 1 ml. of water in a Griffiths homogeniser in the cold room. After grinding the mixture was shaken for 3 hr. and centrifuged at 6000 r.p.m. The supernatant was dialysed against tap water and distilled water successively for three days. All the operations were done in the cold room, and after dialysis the extract was kept at 4° until needed. This extract was given to the gastrectomised rats in amounts of 2·5 ml. divided into three parts as described earlier.

The pig pyloric juice was obtained by collecting the secretion flowing from a fistula; a year previously pyloric juice from this pig had been shown to contain intrinsic factor active in man (Heatley, Jennings, Florey, Watson, Turnbull, Wakisaka and Witts, 1954). It was filtered through a 5/3 sintered glass filter, and the filtrate was dialysed in the same way as the rat stomach extract. Three ml. of this filtrate were given to the gastrectomised rats in the usual way. Three separate batches, collected on different days, were used.

RESULTS.

The absorption of radioactive vitamin B_{12} was measured in four groups of animals: normal rats, gastrectomised rats, gastrectomised rats given an extract of rat stomach, and gastrectomised rats given a dialysed filtrate of pig pyloric juice. The dose of vitamin B_{12} was always approximately 0·005 μg. The results obtained with normal rats are shown in Table I, and those with gastrectomised rats in Table II. In each case the figure given is the amount of vitamin B_{12} excreted in the faeces, expressed as a percentage of the dose.

Normal rats excreted on an average 66·5 per cent of the dose of vitamin B_{12} administered with a range of 52–80 per cent. This is higher than that reported in man (Callender, Turnbull and Wakisaka, 1954) after a dose of 0·5 μg., but in terms of body weight the rat dose is larger. A smaller dose of vitamin B_{12} might have been more completely absorbed but would have necessitated some loss of accuracy in counting.

TABLE I.—*Excretion of Orally Administered Vitamin B_{12} by Normal Rats.*

Rat.	Per cent of dose excreted.	Rat.	Per cent of dose excreted.
1	80	8	73
2	71	9	63
3	59	10	76
4	52	11	55
5	76	12	72
6	61	13	72
7	56	14	65

Mean 66·5. Range 52–80.

TABLE II.—*Excretion of Orally Administered Vitamin B_{12} by Gastrectomised Rats.*

Rat.	Vitamin B_{12} and water. Per cent of dose excreted.		Vitamin B_{12} and rat stomach extract. Per cent of dose excreted.		Vitamin B_{12} and pig pyloric juice filtrate. Per cent of dose excreted.		
	1st trial.	2nd trial.	Batch 1.	Batch 2.	Batch 1.	Batch 2.	Batch 3.
1	96	97	80	81	95	—	—
2	89	93	62	83	79	—	—
3	94	—	68	57	79	90	—
4	90	98	71	61	84	93	70, 69
5	89	95	51	81	76	—	93, 93
6	92	—	—	—	—	83	89
7	93	—	—	—	—	—	94
8	93	—	—	—	—	—	93
Mean	93·8 per cent		69·5 per cent		85·3 per cent		
Range	89–98		51–83		69–95		

Gastrectomised rats were first subjected to experiment 3–4 weeks after operation: by this time they were gaining weight and looked little different from normal animals. Gastrectomised rats excreted on an average 93·8 per cent of the dose of vitamin B_{12}, with a range of 89 to 98 per cent. It is evident that they had lost almost all their ability to absorb vitamin B_{12}.

When gastrectomised rats were given extract of rat stomach along with the vitamin they excreted on an average 69·5 per cent of the dose of vitamin B_{12}, with a range of 51–83 per cent; that is, absorption was restored almost to normal.

Although there was much variation in the amount excreted, the variation was no more than in normal rats.

The results obtained with the filtrate of pig pyloric juice were inconsistent. In some experiments the absorption of vitamin B_{12} was increased, but in the majority there was no evidence of intrinsic factor activity. The mean value for excretion in this group was 85·3 per cent, with a range of 69–95 per cent, a reduction which is probably not significant.

DISCUSSION.

It is clear from these results that normal rats will absorb an appreciable fraction of a small dose of vitamin B_{12} administered orally. It is also clear that resection of the glandular part of the stomach and first part of the duodenum greatly impairs the rat's ability to absorb the vitamin. This suggests that the mucosa of the rat, like that of man, secretes an intrinsic factor necessary for the absorption of vitamin B_{12}. This view is supported by the fact that normal absorption was restored in gastrectomised rats when an extract of rat stomach was fed with the vitamin. Both normal rats, and gastrectomised rats given a rat stomach extract, showed much individual variation in their ability to absorb vitamin B_{12}. We have no explanation for this variation, which is similar to that seen in man.

The results obtained when a preparation of pig pyloric juice was given with the vitamin B_{12} were inconsistent. In some rats absorption of the vitamin appeared to be increased, although never to the degree seen with an extract of rat stomach, but in others there was no detectable effect. The results do not suggest that these variations were due to different degrees of intrinsic factor activity in the three batches of juice, but other explanations can be suggested. There may have been too little intrinsic factor present in 3 ml. of this juice to produce a consistent effect, or a small dose of intrinsic factor may produce a demonstrable effect only when other unknown variables are favourable. Another possibility is that the responses were masked by vitamin B_{12} already present in the pyloric juice; Landboe-Christensen and Wandall (1953) reported that appreciable quantities of vitamin B_{12} were present in pig pyloric juice, and although it was hoped that dialysis would remove most of it there may have been enough left to reduce the absorption of the labelled vitamin. It is also possible that there are species differences in intrinsic factors, and that pig intrinsic factor may not be effective in the rat.

The inflammatory lesions found after death in the remnant of the stomach of the gastrectomised animals which were examined may have influenced their responses to feeding with the vitamin, although there was no sign of ill health at the time of the experiment. In the experiments with the extract of rat stomach any such influence was insufficient to mask the effect of intrinsic factor, but it might have been concerned in the variability of the results.

The changes in the squamous part of the stomach are of interest in relation to the observations of Balfour *et al.* (1950) on totally gastrectomised rats. These authors found that total gastrectomy was followed by sufficient hyperplasia of the squamous epithelium of the oesophagus, which is continuous with that of the upper part of the stomach, to cause obstruction. Balfour *et al.* were able to delay or prevent the development of hyperplasia by administering human gastric

juice, which they considered corrected a specific deficiency resulting from the removal of the stomach. In the one rat of the present series which was killed while still apparently healthy there was very considerable hyperplasia and hyperkeratosis of the epithelium of the remnant of the stomach, although none was seen in the oesophagus. In the stomach of three rats preserved for histological examination some hours after death there was also hyperplasia and hyperkeratosis.

With the evidence to hand it is not possible to state with any assurance what the cause of the ulceration was. It may have been contributed to by mechanical causes or it may have been due to some deficiency depending on the loss of gastric juice. Hoelzel and da Costa (1937–38) ascribed ulceration of the squamous part of the stomach of intact rats to the effects of starvation, a low protein diet or a diet deficient in essential amino acids. In the present series of experiments ulceration occurred in the absence of gastric juice. The phenomenon seems worthy of further investigation.

The method here used did not prove satisfactory for the assay of pig pyloric juice for intrinsic factor; attention has been drawn to possible reasons for this. On the other hand the results obtained with extracts of the animals' own gastric mucosa were satisfactory, and the technique, or adaptations of it, might be of use for the examination of problems relating to the mode of action of intrinsic factor, to its quantitative relationships with vitamin B_{12}, and to the effects of systemic or intestinal disturbances on the absorption of vitamin B_{12}.

SUMMARY.

When normal rats were fed 0·005 μg. of vitamin B_{12} labelled with ^{60}Co the average amount excreted in the faeces was 66·5 per cent of the dose.

After the same dose of labelled vitamin B_{12} gastrectomised rats excreted an average of 93·8 per cent of the dose.

When given an extract of rat stomach along with the vitamin B_{12} the gastrectomised rats excreted an average of 69·5 per cent of the dose.

A dialysed filtrate of pig pyloric juice did not consistently reduce the amount of vitamin B_{12} excreted.

Hyperplasia and hyperkeratosis were observed in the squamous epithelium of the remnant of the stomach. Ulcers, the cause of which is not clear, were also encountered.

(Since this paper was written Latner (1955) has mentioned unpublished observations on normal and gastrectomised rats affording evidence that "intrinsic factor" has some degree of species specificity.)

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