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Influence of Some Specific Group Inhibitors on Rat Intrinsic Factor.

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Abstract.

GRÄSBECK, RALPH. Influence of some specific group inhibitors on rat intrinsic factor. *Acta physiol. scand.* 1959. *45*. 116—121. — The influence of some specific group inhibitors on rat intrinsic factor was studied in order to elucidate the nature of the active structure of intrinsic factor. Iodine and fluorodinitrobenzene inhibited both the vitamin B_{12}-binding capacity and the physiological intrinsic factor activity, whereas *p*-chloromercuribenzoate and *di*-isopropylfluorophosphate lacked effect on either activity. The results add further evidence in favor of the concept that intrinsic factor contains an essential structure which binds B_{12}, and that a phenolic group may be involved in this structure.

A series of previous reports described the influence of group blocking agents and enzymes on the B_{12}-binding capacity (GRÄSBECK 1958 a) and physiological activity (GRÄSBECK 1958 b) of human and hog intrinsic factor material. However, some of the chemical agents being toxic, their action on the physiological activity could not be assayed on patients. The influence of these was therefore studied on gastrectomized rats. For the sake of comparison the influence of iodine was also studied.

Material and Methods.

Adult Wistar rats were operated on in ether anesthesia. The stomach was removed completely and the esophagus was sewn with silk end-to-side to the duodenum; during this stage of the operation the esophagus was intubated with a thin rubber catheter, around which the stitches were placed. After completion of the anastomosis penicillin-streptomycin powder was strewn over the operation area, and 4 ml of physiological saline was injected into the peritoneal cavity during the closing of the wound. After the operation the rats received no other food than sugar water for 4 days, thereafter milk and gradually more solid food. The rats were used for experiments not earlier than 15 days after the operation. Esophageal intubation was performed a few times before the actual experiments to prevent the formation of strictures. The immediate and late operation mortality was about 60 per cent. When not in use for experiments, the rats received 1 μg B_{12} parenterally a week. Experiments were not performed earlier than one week after the last injection.

The intrinsic factor activity was assayed in the following manner: Having fasted overnight the rats were given a light ether anesthesia to ensure quantitative administration of the experimental material. A force-feeding tube was passed down the esophagus and the material was injected with a syringe in the following order: 2 ml of the intrinsic factor preparation (or water when no intrinsic factor was given), 1 ml water containing 10 mμg $\approx$ 10 mμc of Co^{60}-labeled radiovitamin B_{12} ("Merck"), and finally, 1 ml of water to rinse the tube and the syringe. The rats were put into individual metabolism cages and their feces were trapped on wire nets and collected with one or two day intervals for seven days. The feces were put into calibrated "Pyrex" test-tubes, labeled with an ordinary glass-crayon, which leaves a mark visible after incineration, and the tubes were put into an oven kept at 600° C. The incineration of one day's feces took about 24 hours, and thus the material of the whole experimental period was ready to be counted one day after the ending of the collection.

After incineration, the contents of the test-tubes were adjusted to a volume of 7 ml, using dilute hydrochloric acid. The tubes were then counted in a well-type scintillation counter. A tube containing 10 mμg $B_{12}Co^{60}$ was used as a standard; this gave a count about 13 times the background.

A rat intrinsic factor preparation was produced in the following way: During the killing of normal rats for other purposes the glandular parts of their stomachs were cut out and rinsed quickly with distilled water and stored frozen at — 18° C until used after 2—3 weeks. After thawing, the stomachs were washed with ice-cold 1 per cent sodium bicarbonate solution, under which liquid the mucosa was scraped off with a sharp knife. The scrapings were homogenized and dialyzed against distilled water for three days, after which time the material was lyophilized. The dry material bound about 12 mμg B_{12}/mg, as measured by

adding radiovitamin B_{12} followed by dialysis (GRÄSBECK 1956). A few orientating experiments indicated that when 10 mμg B_{12} was administered simultaneously, 10 mg stomach powder elicited a better response than 5 mg and that 20 mg gave about the same response as 10 mg. It was therefore decided to administer 10 mg in the actual experiments. This dose also had the advantage that the binding capacity was almost satured when 10 mμg B_{12} was administered simultaneously.

The effect of the specific group inhibitors was tested in the following manner: All rats were first given only radioactive B_{12}. Next, a control intrinsic factor preparation was given, and finally the treated preparation, equal amounts of both preparations being given. The experiments were usually started immediately after the completion of the previous experimental period. For control purposes, the order of administration of the last two doses was reversed in many cases.

The group blocking treatment was done as follows:

Iodine. Alcoholic I_2 solution was added at 0° C and pH 9 (carbonate buffer) until a final concentration of 0.008 N, the concentration of the stomach extract being 4 mg/ml. Only alcohol was added to the control. Afterwards dialysis at 0° C against pH 9 buffer for 5 hours and later against water to remove free iodine.

Di-isopropylfluorophosphate. To an aqueous solution of 10 mg stomach powder per ml was added in one experiment 0.25 mg and in another one 0.5 mg DFP per ml and the preparation was stirred vigorously for 3 hours. Afterwards dialysis against water for 3 days.

p-chloromercuribenzoate. The substance was first dissolved in a small volume of dilute sodium hydroxide, the pH of which was subsequently adjusted to 7.4. This was added to a solution of 10 mg stomach powder per ml until a final concentration of 1.7 mg PCMB per ml was achieved. The mixture was allowed to stand for 24 hours at 4° C. The visible PCMB crystals were removed by decantation, and afterwards the preparation was dialyzed against pH 8 bicarbonate buffer for three days.

Fluorodinitrobenzene. 30 mg FDNB/ml was added to a solution of 10 mg stomach powder per ml pH 9 phosphate buffer and the solution was stirred vigorously for 3 hours at room temperature. The pH was checked frequently. Afterwards the FDNB was allowed to settle and was removed by decantation. The supernate was dialyzed for 3 days against pH 8.0 phosphate buffer.

The influence of the treatment on the B_{12}-binding capacity was assayed by adding radioactive B_{12}, followed by dialysis as described by GRÄSBECK (1956).

Results.

Effect on B_{12}-binding capacity. Iodine and fluorodinitrobenzene had a clearcut inhibiting effect on the binding capacity, whereas the effect of the two other chemical agents was highly questionable (Fig. 1).

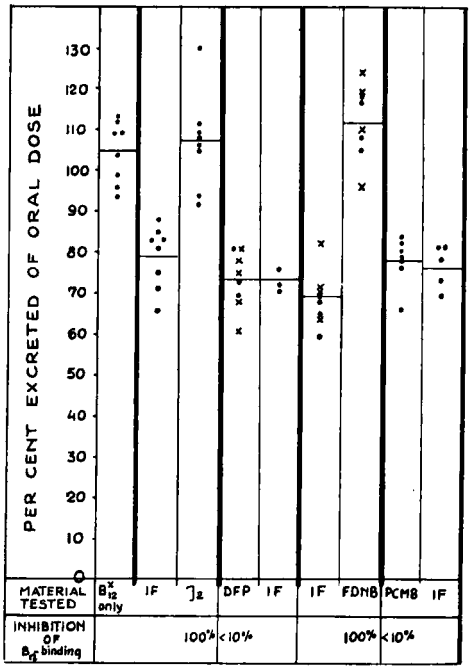

Fig. 1. Influence of iodine (I_2), *di*-isopropylfluorophosphate (DFP), fluorodinitrobenzene (FDNB), and *p*-chloromercuribenzoate (PCMB) on rat intrinsic factor. The horizontal lines indicate mean values, and the crosses and points refer to different batches of treated stomach extract. IF = Control intrinsic factor preparation.

Absorption studies. Absorption of radiovitamin B_{12} alone. 8 normal rats were given 10 mμg $B_{12}Co^{60}$ by mouth. They excreted 35—59 % (mean 41.3 %) of the radioactivity with the feces during seven days after injection. The gastrectomized rats excreted between 93 and 113 % (mean 105 %).

Effect of stomach extract. In four series intrinsic factor (*i. e.* the control samples, which had been subjected to the same treatment as those treated with specific group inhibitors, only the specific treatment being omitted) decreased the excretion of radioactive material considerably. There was no overlapping with the values observed when no intrinsic factor was given.

Effect of specific group inhibitors. When the stomach extract was treated with iodine or fluorodinitrobenzene, no intrinsic factor

activity could be detected and the mean excretion values were even above those observed when no intrinsic factor was given. On the other hand, *p*-chloromercuribenzoate and *di*-isopropylfluorophosphate exerted no demonstrable effect on the intrinsic factor activity.

Discussion.

The excretion values observed when no or inactive intrinsic factor was given are somewhat higher than observed by others (WATSON and FLOREY 1955, TAYLOR *et al.* 1958). This may be due to the fact that total instead of partial gastrectomy was performed and thereby even the last traces of the area producing intrinsic factor were removed. Secondly, the feces were incinerated and counted in a very small volume; this may have increased the influence of natural radioactivity. In repeated experiments these excretion values tended to increase (Fig. 1), apparently due to the fecal excretion of absorbed B_{12}, as observed before (OKUDA, GRÄSBECK and CHOW 1958).

When control intrinsic factor (10 mg stomach extract) was given, the excretion values dropped considerably. This drop was of about the same order of magnitude as observed by others (TAYLOR *et al.* 1958), especially considering that more than 100 % was excreted when no or inactive intrinsic factor was administered.

The absence of inhibiting effect of *p*-chloromercuribenzoate on both the B_{12}-binding and intrinsic factor activities indicates that a sulfhydryl group is not involved in these processes. The effect of iodination, also observed with human and hog material, and fluorodinitrobenzene support the conclusion outlined previously (GRÄSBECK 1958 a, b) that a tyrosyl group may be of importance. The parallel reaction of both the B_{12}-binding capacity and the intrinsic factor activity in the present study adds further evidence in favor of the concept that intrinsic factor contains a structure which binds B_{12} (GRÄSBECK 1956, 1958 a, b).

Summary.

The influence of some specific group inhibitors on rat intrinsic factor was studied. Iodine and fluorodinitrobenzene inhibited both the B_{12}-binding capacity and the physiological intrinsic factor

activity, whereas *p*-chloromercuribenzoate and *di*-isopropylfluorophosphate lacked effect on either activity.

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