Histochemical Demonstration of Mammalian Glucosidase By Means of 3-(5-Bromoindolyl)-β-D-glucopyranoside.* (27014)

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Because of the small amount of glucosidase in mammalian tissue, there is a need for a sensitive substrate for its demonstration in tissue sections. The bromo substituted indole derivitives seemed appropriate because of their relative ease of enzymic hydrolysis as well as the nature of the final reaction product which is highly colored, particulate and insoluble. The 3-(5-Bromoindolyl)-β-D-glucopyranoside was, therefore, synthesized in our laboratory by Dr. Jerome Horwitz and his associates for this purpose in 1958. When this was tested histochemically on mammalian tissue sections, it provided promising results.

This report describes the distribution and localization in tissue of this enzyme and indicates improved synthesis of this substrate.

The principle of the histochemical reaction is similar to that of 5-bromoindoxyl acetate (1,2,3) and is illustrated in Fig.5. The glucosidase in tissue causes hydrolysis of the colorless 3-(5-Bromoindolyl)-β-D-glucopyranoside (I) to form an intermediate, 5-bromoindoxyl (II) which is unstable and is readily transformed to the highly insoluble intensely chromogenic 5,5′-dibromoindigo (III). This is precipitated and can be seen readily at sites of enzymic activity in tissues and cells. The results are quite distinctive and different from those obtained in the study of esterases(1,2,3).

Materials and Methods. The 3-(5-Bromoindolyl)-β-D-glucopyranoside was prepared by Dr. Horwitz and his associates in the organic laboratory of the Detroit Inst. of Cancer Research. The preparation was as follows:

To a cold (−5°C) solution of methanol (100 ml) containing 1.4 g (0.058 g atom) of sodium was added 15 g (0.059 mole) of 5-bromoindoxyl acetate and the greenish brown solution stirred magnetically for 1 hour under an atmosphere of nitrogen. The reaction mixture was then cooled to 0°C and a solution of 24 g (0.059 mole) of acetobromoglucose in 100 ml of methanol was added all at once. The mixture was stirred for 18 hours during which time the reaction mixture was allowed to reach room temperature. The solvent was then evaporated under diminished pressure; water was added to the residue and a stream of air passed into the mixture for *ca* 0.25 hour. The insoluble material was removed by filtration and the filter cake then triturated with 30 ml of warm N,N-dimethylformamide. Norit was added to the extract and the mixture filtered through Celite. The clear filtrate was evaporated to dryness under reduced pressure; the brown residue was suspended in water and filtered: wt. 6.6 g (30% yield), m.p. 245-247° dec. The product was recrystallized from aqueous pyridine to give a colorless solid m.p. 260-261° dec., αD 25°-59° (C=1, 50% aqueous dimethylformamide.)

Analysis:

Calculated for $C_{14}H_{16}BrNO_6$:
C=44.93 H=4.31 N=3.74
Found: C=45.07 H=4.32 N=3.83

The same glucoside was initially obtained from 5-bromoindoxylic acid by a procedure similar to that described by Robertson(4) for synthesis of 3-indolyl-β-D-glucopyranoside. However, the method is tedious and overall yield is poor. Accordingly, the procedure described above is the method of choice. Recently a different procedure for preparation of (I) was reported(5).

Rats, guinea pigs and mice were used for this study. The tissues were cut into blocks 2-4 mm thick, placed on small pieces of filter paper, inserted into glass tubes and immersed in Dewar flasks containing acetone and dry ice at a temperature of about −70°C. The frozen blocks were transferred to a Linderstrom-Lang cryostat and cut at 6μ at −20°C.

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Tissues were affixed to slides and stored at −25°C until ready for use. These slides were placed in a staining rack and immersed immediately into a precooled pretreatment solution (−65°C) consisting of n-butanol, acetone and chloroform (ratio 1:1:1 by volume) in order to remove lipids(6). After remaining in the solvent mixture for 30 minutes the staining rack containing the slides was removed and immediately washed in water with agitation. Three rinses were sufficient to remove the solvent from the tissues. The slides were placed in Coplin jars containing an incubating solution for 3 hours at room temperature (27°C). The composition of the incubating solution was as follows:

4 mg 3-(5-Bromoindolyl)-β-D-glucopyranoside in 1 ml of dimethylformamide.
5 mg PVP in 1 ml of water.
14 ml acetate buffer, 0.2M, pH 5.4.

After incubation the slides were removed from the Coplin jars and rinsed in water and fixed in 40% formaldehyde for 1 minute followed by a thorough rinsing in water. The slides were then stained with Mayer's Haem Alum for 6 minutes, decolorized by 1% aqueous acetic acid and counterstained with a saturated aqueous solution of picric acid for 4 minutes. They were then rapidly passed through a series of graded alcohols to xylene and mounted in balsam. Slides not counterstained were mounted directly in glycerogel or run through alcohols to xylene and mounted in balsam.

Results. In general the enzyme activity was greatest in the preputial glands, duodenum and small intestine with traces in the adrenals, liver, kidney, parotid, pancreas, stomach, spleen, lymph node, epididymis and skin. No reaction was present in the thymus, lung, cerebrum, cerebellum, heart, skeletal muscle, tongue, esophagus, uterus, ovary, oviduct, testes and prostate. This was fairly constant in all species examined with minor variations. Only the outstanding features will be described.

In the preputial gland all the cells of the acinar tissues showed marked enzyme activity, although this varied depending upon age and degeneration of the acini. In small young primordial acini, where the cells were uniform and showed no signs of central degeneration. fine blue granules (III) were dispersed throughout the cytoplasm. In the more mature acini the reaction was more intense and resultant granules larger. In the ductal system, which cannot be conveniently separated from the acini because of their intimate association, degree of intensity was greater with larger granules. The surrounding stratified epithelium was negative (Fig. 1). Areas within the ductal system where definite sebum was present showed an intense activity with accumulation of blue dense granules lying free within the secretory products of the gland (Fig. 2). The stroma of the gland was negative.

The duodenum and small intestine revealed moderate reaction. The large reticulum cells within the villous stalks showed a fairly comparable reaction to that seen in the preputial gland. Only the cytoplasm was positive. The granules were small (Fig. 3,4). A very slight reaction was present in the cells of the intestinal gland. This was different from the esterase reaction with 5-bromoindoxyl acetate which showed intense reaction in these cells but none in the villous stalks. Similar reaction was present in the reticulum cells of the stomach and large intestine, but these cells were extremely sparse.

The adrenals gave a consistently uniform reaction in the cytoplasm of all the cells of the cortex. The granules are larger than those of the liver and kidney and there was an accentuation of these in the glomerular layer. Most of the granules were situated at the peripheral border of the cell. The liver and kidney gave a trace reaction with very fine somewhat elongated granules. Only the cytoplasm was involved. Occasionally the Kupffer cells showed a marked activity. In the kidney only the cytoplasm of the proximal convoluted tubules was involved, but the granules were sparse in comparison with those of the liver. The pancreas revealed fine elongated granules in the cytoplasm of the parenchymatous, but not the insular cells. In the parotid and to some extent the submaxillary gland only the larger ducts showed granules in the cytoplasm. In the spleen only certain reticulum cells in the peripheral sinusoids had granules in their cytoplasm. Not all reticulum cells gave the reaction. Those that did varied in intensity in different animals. A slight diffuse reaction

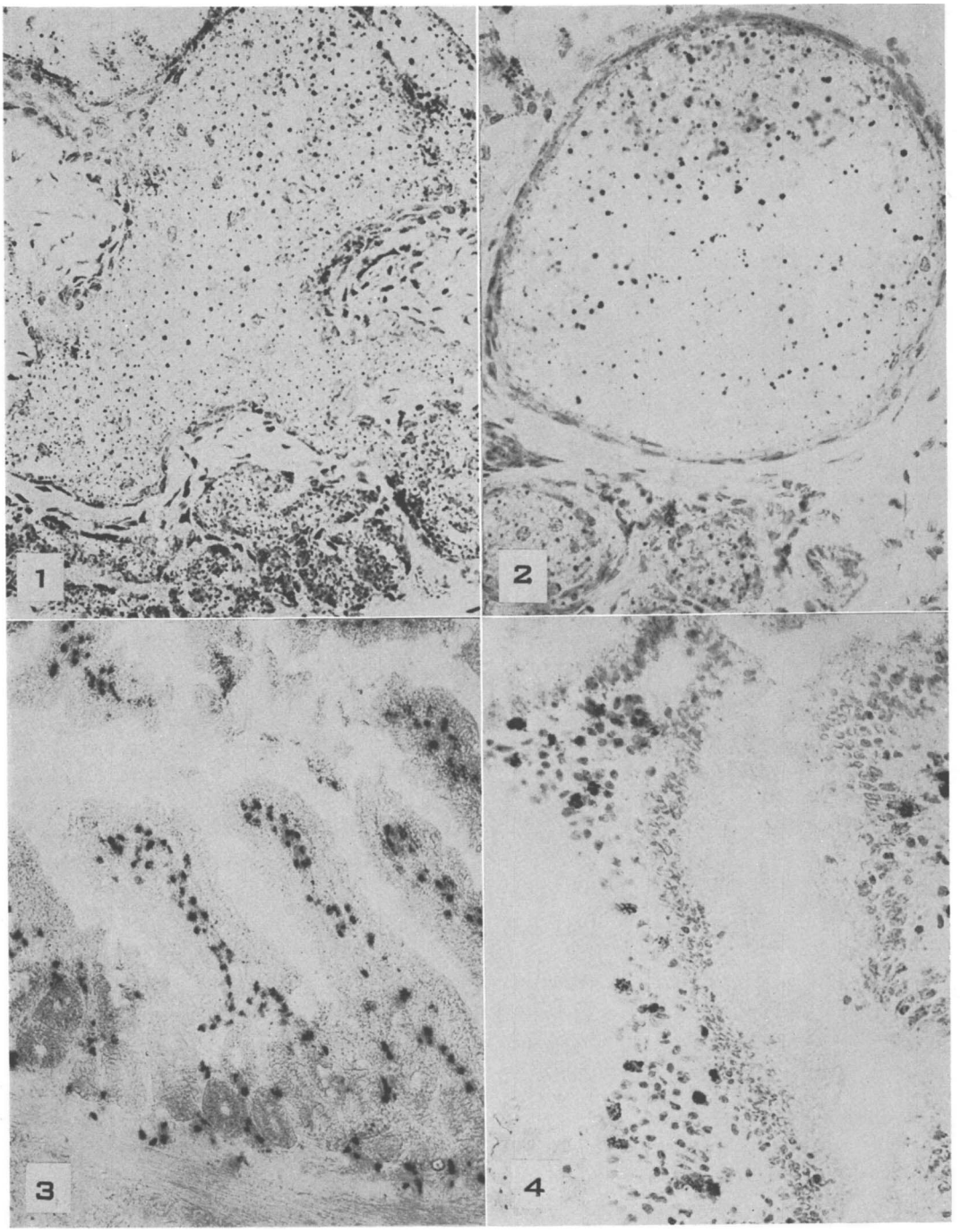

FIG. 1. Preputial gland showing β-glucosidase reaction in acinar cells with varying size granules. Frozen section. Counterstained. X300.

FIG. 2. Preputial gland showing β-glucosidase. Small acini lower left shows discrete enzyme activity in cells. Duct with degeneration showing enzyme activity in sebum. Frozen section. Counterstained. X300.

FIG. 3. Small intestine showing β-glucosidase activity in reticulum cells of villous stalks. Slight activity in mucosal epithelium. Frozen section. Not counterstained. X120.

FIG. 4. Small intestine showing β-glucosidase activity in reticulum cells of villous stalks. Higher power to show granular size in cell cytoplasm. Frozen section. Counterstained. X300.

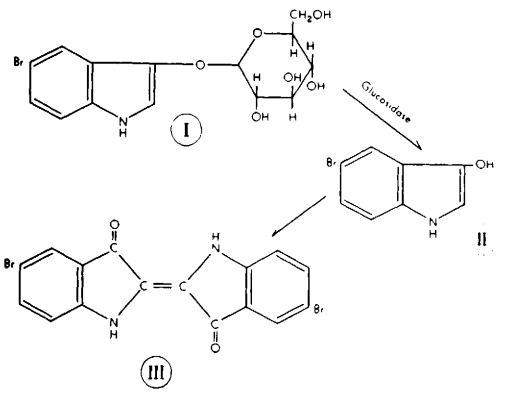

FIG. 5. Substrate Reaction for Tissue Glucosidase.

was present throughout the spleen. The lymph node gave a positive reaction in a few reticulum cells; however, they were not as numerous as those of the spleen. Reticulum cells in the stroma of the epididymis gave a good reaction but they were few in number. The sebaceous glands of the skin gave a good reaction in the cytoplasm and appeared as fairly large, rounded, sparse granules. As a rule, there were only a few granules per cell. but these were consistent.

Characterization of Tissue Glucosidase. When slides with the tissue were placed in a water bath (58°C) for 1 hour and then incubated in the substrate solution. no hydrolysis of the substrate (I) occurred. indicating that the enzyme was destroyed by this process. When the potassium salt of ferri-ferrocyanide (0.05M) was added to the substrate solution and the slides incubated no hydrolysis took place. When 2% formalin was placed in the incubating media for 3 hours. no hydrolysis occurred and the final reaction was negative. The enzyme is sensitive to formalin. When copper sulphate was added to the substrate solution (1 ml of 0.03M) and the slides incubated, no reaction occurred.

Boiled saccharate was used to produce saccharo-1-4-lactone which is a strong inhibitor of glucuronidase(7). This was made up fresh. cooled and added to the incubating solution (1 ml of 0.05M). A series of slides incubated in this solution showed no inhibition of hydrolysis (I) and the final reaction product was the same as in the slides incubated without saccharate.

A high concentration of the salt (I) caused inhibition of the reaction, so a series of 9 substrate solutions were set up containing graded final concentrations of (I) from 0.006 M to 0.00007M. Optimal concentration was found to be 0.0006M. This inhibitory effect has been noted for intestinal β-glucosidase using phenol substrates(8).

To determine an optimal pH, a series of 8 substrates were prepared with pH's ranging from 2.0 to 8.8. No reaction occurred below a pH of 5.0 or above 6.2. Optimum pH was 5.4.

Discussion. Although β-glucosidase is an enzyme which occurs mainly in plants and fungi, small amounts occur in mammalian tissue. Recently, 2 investigators(9,10) have made surveys of its occurrence in mammalian tissue using homogenate methods. The substrates used were 6-bromo-2-naphthyl-β-D-glucopyranoside and 4-methylumbelliferone-β-glucoside, respectively. In the former(9) the glucosidase splits off the glycone leaving the aglycone which is coupled with tetrazotized diorthoanisidine under alkaline conditions. The colored final product was measured colorimetrically. Attempts were made to use this method histochemically on tissue sections, but an entirely satisfactory result was not achieved. The latter(10) employed an extremely sensitive method using 4-methylumbelliferone-β-glucoside. This was prepared by the authors fresh daily as it decomposed readily. The glucosidase in the tissue split off the glucose leaving the aglycone which was measured fluorometrically.

In both of these studies the intestine had a high activity. Liver and kidney also showed activity. In the former study the guinea pig pancreas was very active(9). In our histochemical investigations we did not find this disproportion. The preputial gland was not studied in either series. It should be appreciated that extremely small amounts in various tissues were demonstrated by these studies.

In our studies definite enzyme activity was demonstrated in the preputial gland, small intestine and the adrenal gland. In other organs such as the liver and kidney only trace amounts were demonstrated. In the reticuloendothelial cells of the sinus reticulum of the spleen and lymph node good reactions were present. This, however, was variable as not

all of the reticulum cells showed the same intensity. Reticulum cells of the thymus were negative. Under certain conditions the Kupffer cells of the liver showed a strong reaction. The conditions responsible for this variation are being studied.

Summary. The 3-(5-Bromoindolyl)-β-D-glucopyranoside (I) was prepared in our laboratory for histochemical study of tissue β-glucosidase. The final reaction product following incubation of tissue sections was 5,5′-dibromoindigo which is highly chromogenic and precipitates in cells and tissue at the site of enzymic activity. The enzyme is readily destroyed in tissue by ferri-ferrocyanide and formalin, which is not the case with non-specific esterase or glucuronidase. Saccharo-1-4-lactone, which has an inhibitory effect on β-glucuronidase, does not affect β-glucosidase. "Substrate inhibition" can also be demonstrated. Optimum pH is 5.4. The β-glucosidase can be demonstrated best in the preputial gland where activity is high. The older acini of the gland showed more intense reaction. Reticulum cells within the villous stalks of the small intestine showed a good reaction. Reticulum cells of the spleen, lymph node and Kupffer cells showed a varied reaction. Extremely small amounts are present in the liver and kidney.

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Diagnosis of Human Allergy Utilizing Passive Skin-Sensitization in the Monkey *Macaca irus.* (27015)

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If blood serum from an actively allergic individual is injected into the skin of a nonallergic volunteer the skin of the recipient may become sensitized in the immediate area of the serum injection. This phenomenon is referred to as passive sensitization. The passively sensitized skin site will often exhibit an inflammatory reaction with wheal formation when injected with a minute amount of the substance(s) causing allergy in the serum donor. This particular direct local application of the phenomenon of passive cutaneous anaphylaxis (P.C.A.) in humans is referred to as the "Prausnitz-Küstner test" or the "P.-K. test" (1). The P.-K. test has been used extensively as a diagnostic aid in identifying the substances responsible for allergies, and is especially useful in allergies of infants, debilitated children and severely allergic older patients.

The P.-K. test presents the grave danger of transfer of serum hepatitis infection to the human volunteers. Because of this danger of infection we avoided the use of the technique in testing the sera of those factory employees showing signs of sensitivity to castorbean dust.

Ovary(2) has discussed the phenomenon of passive cutaneous anaphylaxis in guinea pigs and other lower animals. He suggested that a colloidal dye such as Evans Blue be injected

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