In mice sensitized to horse serum, β-glucuronidase prevents the increased sensitivity which results from a second dose of antigen given one week before challenge by pinnal anaphylaxis. β-glucuronidase loses this activity with age or in the presence of gelatin. In both cases glucose will restore the immunological blocking activity of the enzyme. These results suggest a reason for variability in the hyposensitizing activity of the different samples of enzyme used in earlier clinical and animal experiments.

ENZYME-POTENTIATED HYPOSENSITIZATION

Effects of glucose, glucosamine, N-acetylamino-sugars and gelatin on the ability of F β-glucuronidase to block the anamnestic resposen to antigen in mice.

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Introduction

FOLLOWING THE discovery of the ability of the enzyme β glucuronidase to potentiate the hyposensitizing effect of small doses of antigen in the treatment of clinical allergy[1] the first paper in this series described a similar behaviour of the enzyme in three different models of experimental anaphylaxis. [2] However, the potentiating effect of different samples of β -glucuronidase has been found to vary, so that it was impractical to proceed to further clinical trials and the results of animal experiments were not consistent.

This paper is a study of the source of the variability and describes the effect of glucose, glucosamine, certain N-acetylamino-sugars and gelatin on the ability of the enzyme to modify the anamnestic response to a second dose of antigen in mice.

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Method

The materials and methods for mouse pinnal anaphylaxis were previously described[2] except that all samples of β -glucuronidase had been passed through a column of polyacrilamide gel (Bio-Gel P-6 Bio-Rad Laboratories) to remove the sorbitol which was added to the enzyme as a stabilizer during commercial freeze-drying. For this purpose the buffer previously described was employed without phosphate. The protein void from the column was saved. There was no appreciable loss of total β -glucuronidase activity. Sterility was maintained by passage through Millipore filters. Gelatin was obtained from Armour Pharmaceutical Co. Ltd. and from E. Merck A. G. . Solutions were stored at —20° C before use.

The *in vitro* assay of β-glucuroni-

TABLE I.

Pinnal anaphylaxis in groups of 35 mice. Activation by glucose 0.1 μg of the ability of aged β-glucuronidase 10U. to block the anamnestic response to a subcutaneous dose of 1 μg horse serum given 3 weeks after initial sensitization and 8 days before challenge.

Treatment	Mean Area Blued mm□
Saline	29
Horse serum	36
Horse serum + β-glucuronidase	40*
Horse serum + β-glucuronidase + glucose	30*

*Significance: p = <0.02

TABLE II.

Pinnal anaphylaxis in groups of 21 mice. Effect of gelatin 1.0 ng on the ability of β-glucuronidase 10U to block the anamnestic response to a subcutaneous dose of 1μg horse serum given 3 weeks after initial sensitization and 8 days before challenge.

Treatment	Mean Area Blued mm□
Saline	46
Horse serum	56
Horse serum + β-glucuronidase	47*
Horse serum + β-glucuronidase + gelatin	62*

*Significance: p = <0.02

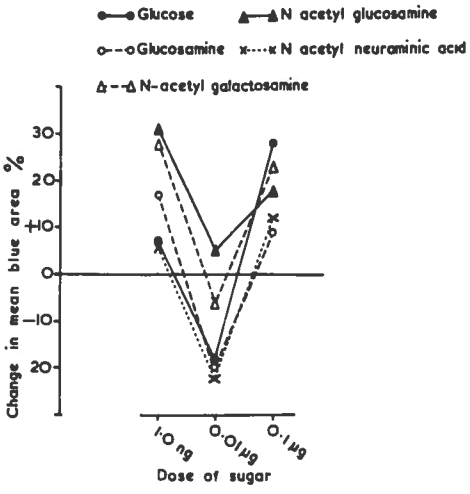

Figure 1. Mouse pinnal anaphylaxis: groups of seven mice sensitized with 250 μg horse serum. Treated after three weeks with 1 μg horse serum + 10U β-glucuronidase + various doses of sugars and challenged eight days later. Results expressed as percentage of result after treatment with antigen + enzyme.

dase activity using phenolphthalein glucuronide (Sigma Chemical Co.) was carried out by the method described in Sigma Technical Bulletin No. 105 (1958). However, the experiments were duplicated in the buffer used for the animal work which has a pH of 5·6. Further experiments assessed the effect of glucose and N-acetyl glucosamine on the plasma inhibitor of β-glucuronidase, using the same buffer with 6.5 percent defibrinated human serum.

Results

In an earlier study, it was noticed that batches of β-glucuronidase which had aged lost their hyposensitizing activity and often had the opposite effect. Table I shows the effect of such a sample of enzyme, freeze-dried and stored at - 80°C for 18 months, on the anamnestic response of mice.

The addition of this aged enzyme to the second dose of antigen results in a repeatable, but not significant, increase in anaphylactic sensitivity. When glucose 0.1 μg per mouse is added to the treatment mixture of antigen and enzyme shortly before injection, the ability of the enzyme to block the anamnestic response is revealed.

If concentrations of glucose greater and less than 0.1 μg are added to the enzyme and antigen mixture, they induce an increase in anaphylactic sensitivity, but if glucose and enzyme are allowed to interact *in vitro* at room temperature for 1 hour before the mixture is injected, the critical dose of glucose for blockade of the anamnestic response is reduced tenfold to 0.01 μg/mouse.

This ability to modify the effect of β-glucuronidase is not peculiar to glu-

cose; the action is shared by many other sugars. Figure 1 shows the striking similarity of the dose-response curves obtained with varying concentrations of glucose, glucosamine, N-acetyl glucosamine, N-acetyl galactosamine and N-acetyl neuraminic acid. The results are expressed as percent change of the effect of antigen and enzyme without added sugar. The five dose-response curves shown are taken from consecutive experiments. In this series, the sample of β-glucuronidase used had a slight hypersensitizing effect when not modified by sugar.

Table II shows the effect of adding gelatin 1.0 ng per mouse to the treatment mixture shortly before injection. In this experiment the β-glucuronidase had not been stored for a long period, and possessed the ability to block the anamnestic response without the addition of glucose. Gelatin reverses the action of the enzyme so that it induces hypersensitization. This result was reproduced in five consecutive experiments. The gelatin from Merck had a consistent effect over a wide dose range. The gelatin from Armour also blocked the desired effect of the enzyme at 1.0 ng per mouse, but at higher concentrations its dose-response curve followed a pattern which resembled those of the sugars in Figure 1.

Table III shows that glucose can restore the ability to block the anamnestic response to a mixture of enzyme and gelatin. In further experiments glucose was shown to antagonize the effect of both samples of gelatin.

Experiments *in vitro* have been carried out to assess the effect of glucose, N-acetyl glucosamine and gelatin on the activity of the enzyme against phenolphthalein glucuronide. At concentrations of 1 percent, glucose and N-acetyl glucosamine inhibit the enzyme's activity by 15 percent and 36 percent respectively. Greater dilutions have little effect. Defibrinated human serum 6.5 percent reduces the enzyme activity to 20 percent of the control, but N-acetyl glucosamine 1 percent restores the activity to 33 percent of control. Weak antagonism of the plasma inhibitor of β-glucuronidase is seen with concentrations of N-acetyl glucosamine down to 0.01 percent. Glucose does not behave in this way. Gelatin has no influence on the activity of the β-glucuronidase used in the present experiments.

TABLE III.

Pinnal anaphylaxis in groups of 8 mice. Effect of glucose 0.1 μg and gelatin 1.0 ng on the ability of β-glucuronidase 10U to block the anamnestic response to a subcutaneous dose of 1 μg horse serum given 3 weeks after initial sensitization and 8 days before challenge.

Treatment	Mean Area Blued mm□
Saline	40
Horse serum	51
Horse serum + β-glucuronidase	41
Horse serum + β-glucuronidase + gelatin	57*
Horse serum + β-glucuronidase + gelatin + glucose	40*

*Significance: $p = < 0.05$

Discussion

These experiments identify an obstacle which has prevented further progress in the study of enzyme-potentiated hyposensitization for a number of years. Different samples of β-glucuronidase have widely differing hyposensitizing effects. Some batches of *Patella vulgata* enzyme have no hyposensitising activity even when fresh, and all batches will slowly lose their ability in this direction on storage, although freeze-dried and kept at - 80°C. Nevertheless, the activities of different samples against phenolphthalein glucuronide do not differ greatly, and their degrees of purity do not vary sufficiently to account for their great differences in hyposensitizing ability.

The two most likely explanations of these facts are either that a contaminant

of the β-glucuronidase is really responsible for its hyposensitizing effect, or that contaminants, perhaps other enzymes, interfere with the desired activity. The latter explanation commended itself early in this work when Robinson and Stirling[3] examined samples of ovine testicular hyaluronidase and found that batches which possessed hyposensitizing activity contained more β-glucuronidase than N-acetyl glucosaminidase. In batches with no hyposensitizing activity the proportions were reversed.

The first possibility has also been considered since Mowbray and his coworkers[4,5] have reported that polyribonuclease is capable of inducing immunological tolerance, and the β-glucuronidase used in the present work was contaminated with ribonuclease. There are, however, a number of differences between the behavior of Mowbray's enzyme preparation and the β-glucuronidase used in the present experiments: First, in Mowbray's work, tolerance induction by polyribonuclease could not be achieved unless it was given some hours before or after the antigen. Administration by a protocol similar to that used for β-glucuronidase was ineffective. Second, the optimal dose of purified polyribonuclease was 200 μg per mouse. This compares with 5 μg per mouse for impure β-glucuronidase. Third, very small doses of sugars would not be expected to influence the behavior of polyribonuclease, but suggest that a carbohydrase or a carbohydrate receptor are more likely to be involved.

Newsome[6] has found that the tolerance-inducing properties of polyribonuclease preparations were due to a product of contaminating bacteria and not to the enzyme itself. The β-glucuronidase used in the present work was purchased already freeze-dried, and was sterile at all stages thereafter. Original contamination of the molluscan digestive glands is a possibility which has not been excluded. Nevertheless, the difference in behaviour between β-glucuronidase and the agent investigated by Mowbray strongly suggest that they are not identical.

The work reported here shows that the ability of β-glucuronidase from Patella vulgata to block the anamnestic response in mice is activated by the presence of extremely small quantities of sugars. In earlier work, β-glucuronidase was used with sorbitol 20 percent by weight as stabilizer. It seems possible that the variability of results at this time may have been partly due to contamination of the commercial sorbitol with glucose or other sugars. At the above concentration, pure sorbitol does not cause β-glucuronidase to block the anamnestic response in mice, and the ability of added glucose to elicit this activity from aged β-glucuronidase was first noted in four consecutive mouse experiments using enzyme which contained sorbitol.

Figure 1 shows that reversal of the effect of the enzyme and antigen mixture can be brought about by varying the dose of sugar employed. The unusual dose-response relationship will be considered more fully in the next publication in this series.

In vitro, neither glucose nor N-acetyl glucosamine altered the enzyme's hydrolysis of phenolphthalein glucuronide except in concentrations of 1 percent, when some suppression of enzyme activity was noted. This contrasts with the 10,000-fold lower dose of either required to potentiate the enzyme's ability to block the anamnestic response in the mouse.

The inhibitory effect of plasma on the enzyme's activity *in vitro* was antagonized to a small degree by N-acetyl glucosamine, again only at high concentrations. Glucose did not possess this action. Since glucose and N-acetyl glucosamine have similar effects on the immunological behaviour of the enzyme,

it is inferred that for this role, any action on the plasma inhibitor of β-glucuronidase is immaterial.

Gelatin is an activator of purified β-glucuronidase *in vitro*.[7] At the low degree of purity of the enzyme used in the present experiments, gelatin had no effect on *in vitro* activity against phenolphthalein glucuronide. *In vivo*, gelatin's ability to alter the hyposensitizing effect of the enzyme was studied since it seemed likely that other denatured proteins might act in the same way. In common with many proteins, gelatin is known to contain many hexose groups. It is suggested that the difference in behaviour of the two samples which were tested was due to some difference in the distribution of the hexose groupings on the surface of the molecules.

Molluscan β-glucuronidase from Helix pomatia has been prepared with an activity of 120,000 U/mg,[8] by which standard the preparation used in the present work contained about 98 percent of extraneous protein. The precipitation procedure used in the preparation of the enzyme, and ageing after freeze-drying might both produce variable amounts of altered contaminants capable of exerting a gelatin-like effect. Since gelatin has this action at such low concentrations, it is clear that a very high degree of purification of the enzyme would be necessary to obviate the possibility. That the enzyme's loss of hyposensitizing activity on storage is in reality due to an alteration of a contaminant is suggested by the ability of glucose to restore the anti-anamnestic effect to aged enzyme as well as to reverse the action of gelatin.

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