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Castanospermine inhibits α-glucosidase activities and alters glycogen distribution in animals

(metabolism/lysosomes/vesicles)

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ABSTRACT **Castanospermine, an inhibitor of α-glucosidase activity, was injected into rats to determine its effects *in vivo*. Daily injections of alkaloid, at levels of 0.5 mg/g of body weight, or higher, for 3 days decreased hepatic α-glucosidase to 40% of control values, whereas α-glucosidase in brain was reduced to 25% of control values and that in spleen and kidney was reduced to about 40%. In liver, both the neutral (pH 6.5) and the acidic (pH 4.5) α-glucosidase activities were inhibited, but the former was more susceptible. On the other hand, β-*N*-acetylhexosaminidase activity was elevated in the livers of treated animals, whereas β-galactosidase activity was unchanged and α-mannosidase activity was somewhat inhibited. Livers of treated animals were examined by light and electron microscopy and compared to control animals to determine whether changes in morphology had occurred. In treated animals fed normal rat chow, the hepatocytes were smaller in size and simplified in structure, whereas the high-glucose diet lessened these alterations. Furthermore, in those animals receiving castanospermine at 1.0 mg or higher per g of body weight for 3 days, there was a marked decrease in the amount of glycogen in the cytoplasm, while a large number of lysosomes were observed that were full of dense, granular material. That this dense material was indeed glycogen was shown by the fact that it disappeared when blocks of fixed tissue were pretreated with α-amylase. Glycogen levels in liver, as measured either colorimetrically or enzymatically, were somewhat depressed at the higher levels of castanospermine.**

Castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizine) is an indolizine alkaloid that is found in the seeds of the Australian tree *Castanospermum australe* (1). These seeds have been reported to be toxic to animals and to cause various symptoms, including gastrointestinal upset. We recently found that this alkaloid was a potent inhibitor of lysosomal α- and β-glucosidase but had no effect on a number of other glycosidases (2). In addition, castanospermine also inhibits the neutral glucosidase(s) that participates in the processing of the oligosaccharide chains of the N-linked glycoproteins (3). Thus, in cultured mammalian cells, castanospermine prevents the formation of complex chains and gives rise to glycoproteins having mostly $Glc_3Man_{7-9}GlcNAc_2$ structures.

Since glucosidases are very important enzymes in a number of metabolic sequences, including glycogen degradation, we were interested in determining whether this alkaloid would affect glycogen metabolism if injected into animals. Based on preliminary experiments, we postulated that castanospermine would inhibit the lysosomal α-glucosidase and produce a lysosomal block leading to the abnormal storage of glycogen. The data presented in this paper show that this alkaloid markedly depressed the activity levels of α-glucosidase in liver and other tissues. Coupled with this decreased activity was the finding that the livers of rats given this alkaloid for several days had lowered levels of cytoplasmic glycogen, while the lysosomes were filled with dense granular material that looked like glycogen particles. Treatment of liver sections with amylase led to the complete disappearance of this granular material. Thus, this alkaloid should be of interest because it causes an abnormal distribution and storage of glycogen in experimental animals. Such studies may also be important in understanding the mechanism of toxicity of castanospermine and other alkaloids.

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EXPERIMENTAL METHODS

Castanospermine was isolated in 0.3% yield from the seeds of *C. australe* and crystallized several times from ethanol. The alkaloid was dissolved in pyrogen-free water and sterilized by filtration for injection into animals. Young Sprague–Dawley rats were injected intraperitoneally with various amounts of alkaloid, ranging from 0.1 mg to 2.5 mg/g of body weight. The animals were weighed daily. For most of the studies described here, animals were given daily injections of the drug over a 3-day period. However, other regimens of injections were also tried, including time-course studies. On the fourth day (after three injections), the animals were sacrificed by decapitation, and the brain, heart, spleen, kidney, and liver were removed for assay of various enzymes and, in some cases, for microscopic examination and glycogen determinations. Control animals were given daily injections of saline and were kept and handled under identical conditions to treated animals.

In the first experiments with castanospermine, animals were allowed to eat the normal rat chow at will. We found that some animals given high doses of castanospermine (2 mg/g of body weight) developed diarrhea and had high bacterial counts in their intestines. Since we found that castanospermine inhibited intestinal sucrase and maltase, the above symptoms suggested that these animals might be suffering from a maltose or sucrose intolerance (unpublished results). To be certain that any observed effects were not due to lack of nutrients, a special diet was prepared that contained protein, vitamins, and glucose but was devoid of sucrose and starch. Animals treated with castanospermine and maintained on this diet did not exhibit any gastrointestinal symptoms and did not have diarrhea. Furthermore, the effects of the alkaloid on hepatic enzymes and on glycogen distribution were the same in animals given either diet.

Tissues from control and experimental animals were weighed and homogenized in a 10-fold volume (wt/vol) of buffer (0.1 M citrate/phosphate, pH 6.8/10 mM EDTA/50 mM KCl) using a glass–Teflon homogenizer. The crude homogenate was centrifuged at 500 rpm for 2 min to remove cellular debris and connective tissue. The resulting supernatant was collected and aliquots were assayed for the various glycosidases (i.e., α-glucosidase, α-mannosidase, β-*N*-acetylhexosaminidase, β-glucosidase). For these assays, sever-

al different aliquots of each tissue extract were incubated with the appropriate *p*-nitrophenyl glycoside substrate in either phosphate, citrate, or citrate/phosphate buffers at pH values from 3.5 to 7.0. Incubations were done at 37°C for various times and the release of *p*-nitrophenol was measured at 410 nm. Protein was determined by the method of Lowry *et al.* (4).

Portions of each tissue were fixed for light and electron microscopy. Thin sections of tissue were immersed in phosphate-buffered formalin or in alcoholic Carnoy's solution for light microscopy. Small pieces of tissue for electron microscopy were removed from representative areas, diced into 1-mm cubes, and fixed for 2 hr by immersion in cold 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer at pH 7.3. The tissue blocks were next rinsed in several changes of buffer and then postfixed in buffered osmium tetroxide for 2 hr. After appropriate rinses and stepwise dehydration, the tissue blocks were infiltrated with propylene oxide and then with resin monomer. The tissue, when fully infiltrated with complete monomer, was placed in BEEM capsules and polymerized in ovens at 70°C. The embedments were cut on a Sorvall MT 5000 with glass or diamond knives. Sections were stained with aqueous alkaline lead citrate and uranyl acetate. Transmission electron microscopy was performed with a Philips 301 or a Joel 100C.

Glycogen was isolated from fresh rat liver by the method of Walaas and Walaas (5) as modified by Hung and Menahan (6). By using purified rabbit glycogen as a standard (type III, Sigma), the amount of glycogen precipitated from each experimental liver was estimated by quantitating the total glucose liberated with either an enzymatic hydrolysis or acid hydrolysis. Glucose was determined by the Nelson procedure (7).

RESULTS AND DISCUSSION

The rate of weight gain for these young, actively growing, female rats was decreased in proportion to the dose of alkaloid. This arrest of growth was severe in animals given high doses of castanospermine (2 mg/g of body weight) and fed the normal rat chow. Such animals developed severe diarrhea. However, animals on the high-glucose (starch and sucrose-free) diet experienced better weight gains, even at high doses of the alkaloid, and did not develop diarrhea.

At autopsy, the weights of livers of treated animals were generally decreased in proportion with the body weight. In animals given high doses of alkaloid, the body hair became slightly discolored (off-white) and lost its sheen and neatly combed appearance. In addition, these animals became hyperactive, frequently overreacting to noises.

The activities of various glycosidases were examined in the livers of animals treated for 3 days with different amounts of castanospermine (Fig. 1). The curves in the Fig. 1 *Upper* show the activity of liver α-glucosidase in control and treated animals. The activities of both the neutral (pH 6.5) and the acidic (pH 4.5) α-glucosidases decreased with increasing doses of castanospermine, but the neutral enzyme was more susceptible to inhibition. Measurements of enzymatic activity were done under linear conditions with respect to both time of incubation and amount of enzyme, and inhibition was evident throughout the time course. Even a single injection of castanospermine (at 1 mg/g of body weight) reduced liver α-glucosidase activity about 30%, whereas a second injection, given at the same dosage level 24 hr later, reduced activity another 15–20%. In animals given one or two injections of alkaloid and then allowed to stay for 3 or 4 days without further treatment, there was a partial return of α-glucosidase activity in various tissues. Thus, in one experiment, liver α-glucosidase returned to 85% of normal in 4 days while kidney α-glucosidase was 70% of control

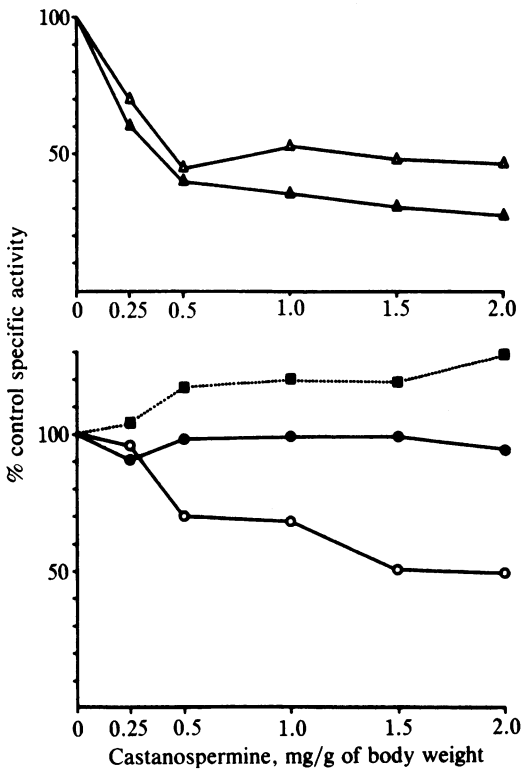

FIG. 1. Enzymatic activities of liver homogenates. Each point represents an average of the activity observed in homogenates from five separate animals given the same dose of castanospermine. Activity is expressed as % of control specific activity. Incubation mixtures contained 0.5–1.0 mg of homogenate protein, 10 mM substrate (the appropriate *p*-nitrophenyl glycoside), and 0.1 M citrate/phosphate buffer (at specified pH) in a total volume of 0.5 ml. Mixtures were incubated for 30–60 min at 37°C. (*Upper*) α-Glucosidase activity measured at pH 4.5 (△) and pH 6.5 (▲). (*Lower*) Activity of β-*N*-acetylglucosaminidase (■), β-galactosidase (●), and α-mannosidase (○).

values. It is not clear at this stage whether these decreases in enzyme activity are due to inhibition of existing enzyme by castanospermine or whether there is an actual decrease in the amount of enzyme. Attempts to reisolate castanospermine from the livers or from other tissues (i.e., spleen, kidney, heart, brain, intestine) of treated animals were unsuccessful. Whether the alkaloid is metabolized to other compounds, is excreted in the urine and feces, or is present in other tissue that have not been examined remains to be established.

The curves in the Fig. 1 *Lower* show the activities of three other glycosidases in the livers of treated and control animals. The activity of β-*N*-acetylhexosaminidase was elevated in livers of treated rats, and this increase in activity was observed at various doses of alkaloid. The activity of β-galactosidase remained essentially the same, regardless of the alkaloid dose. Like α-glucosidase, the activity of α-mannosidase also decreased, but at higher doses of castanospermine. The reason for the elevation or inhibition of these other glycosidases is not clear at this time. However, castanospermine is an inhibitor of glycoprotein processing (3) and, since these enzymes are probably N-linked glycoproteins, it could be related to their targeting to lysosomes—that is, if the inhibitor prevents phosphorylation of the oligosaccharides, the recognition signal for insertion into lysosomes would be absent. Results similar to those shown in Fig. 1 were also obtained with treated and control animals on the high-glucose (sucrose and starch-free) diet. Thus, the changes observed in glycosidase activities are likely effects of the alkaloid rather than of nutritional factors.

Table 1. α-Glucosidase activity in various tissues

Castanospermine, mg/g of body weight	% control*			
	Liver	Brain	Kidney	Spleen
0	(0.373)	(0.258)	(0.334)	(0.202)
0.01	94	81	82	80
0.05	86	55	76	78
0.25	50	33	48	50
0.50	47	29	49	49
1.00	40	24	40	37

Incubation conditions: 0.5–1 mg of homogenate protein, 2 mM *p*-nitrophenyl-α-glucoside, 0.1 M sodium citrate buffer (pH 6.5); total volume, 0.5 ml. The mixture was incubated for 45 min at 37°C.
*Numbers in parentheses represent the specific activity (OD units/hr per mg) of the control homogenates from the various tissues. All other activities are expressed as % control.

The activity of α-glucosidase as a function of castanospermine dose was also examined in several other tissues (Table 1). The activity was also markedly inhibited in brain, kidney, and spleen. In fact, brain was the most sensitive tissue of those examined, with activity being depressed to 24% of control values at 1 mg/g of body weight. Table 1 shows the activities of the neutral α-glucosidase (pH 6.5) of various tissues. However, the activities of the acidic enzyme in these tissues were also inhibited to about the same extent as the neutral enzyme.

Since the acidic, lysosomal α-glucosidase is known to be involved in glycogen metabolism, we examined the livers of animals treated with castanospermine to determine whether any changes in glycogen levels or distribution could be observed. Light microscopy of livers from animals receiving the higher doses of drug (i.e., 1 mg or higher per g of body weight) and fed the normal rat chow consistently revealed that the hepatocytes were smaller in size than those of normal animals. However, in animals given the high-glucose diet, the hepatocytes appeared to be closer to normal size. We assume that on the high-glucose diets, the animals are better able to metabolize dietary carbohydrate. In both diets, however, periodic acid–Schiff reagent (PAS)-stained hepatocytes from treated animals revealed an unusual distribution of glycogen. In the normal cells stained with PAS, the glycogen appeared to be spread throughout the cell, whereas in treated animals, it was much more punctate in appearance.

To more clearly determine the distribution of the glycogen in these cells, livers from normal and treated animals were examined by transmission electron microscopy. Fig. 2 shows a representative section from the liver of a control animal, whereas Fig. 3 is that of a castanospermine-treated (2 mg/g of body weight) animal. The liver from the control animal shows the usual abundant rough and smooth endoplasmic reticulum, mitochondria, and glycogen. The cytoplasmic glycogen was present in both the α-form (aggregated particles) and in the β-form (individual particles). In contrast, the intracellular distribution of glycogen was unusual in the animals given castanospermine (Fig. 3). In this case there was a marked decrease in the amount of nonvesicular glycogen in the cytoplasm. Instead, these hepatocytes contained large numbers of vesicles that were filled with a dense granular material. These vesicles, which are thought to be lysosomes, also contained myelin figures. To show that this dense granular material was glycogen, fixed liver tissue from treated animals was incubated with α-amylase (Sigma) and then processed for electron microscopy. Fig. 4 shows that this treatment almost completely removed the dense granular material, while the myelin figures and cellular debris remained within the vesicles. Thus, the dense granular material contained within the vesicles does appear to be glycogen. These results on the abnormal distribution of glycogen were essentially the same with treated animals regardless of the diet, although animals on the high-glucose diet had more intrahepatic glycogen in all experimental and control groups.

Since the electron microscopy revealed a change in the distribution of glycogen upon alkaloid treatment, we examined the levels of glycogen in the livers of control and castanospermine-treated animals. These results are shown in Fig. 5. Livers were removed immediately after decapitation and placed in 30% KOH and heated at 95°C for 2 hr. At doses of castanospermine up to 1 mg/g of body weight, there was no effect upon levels of hepatic glycogen. However, at the higher doses of alkaloid, there did appear to be some decrease in hepatic glycogen levels. As indicated above from the electron microscopy, most of the glycogen is vesicle-bound. A preliminary experiment on the sizing of the glycogen by gel filtration did not reveal any gross differences in molecular weight between control and treated animals, although such a

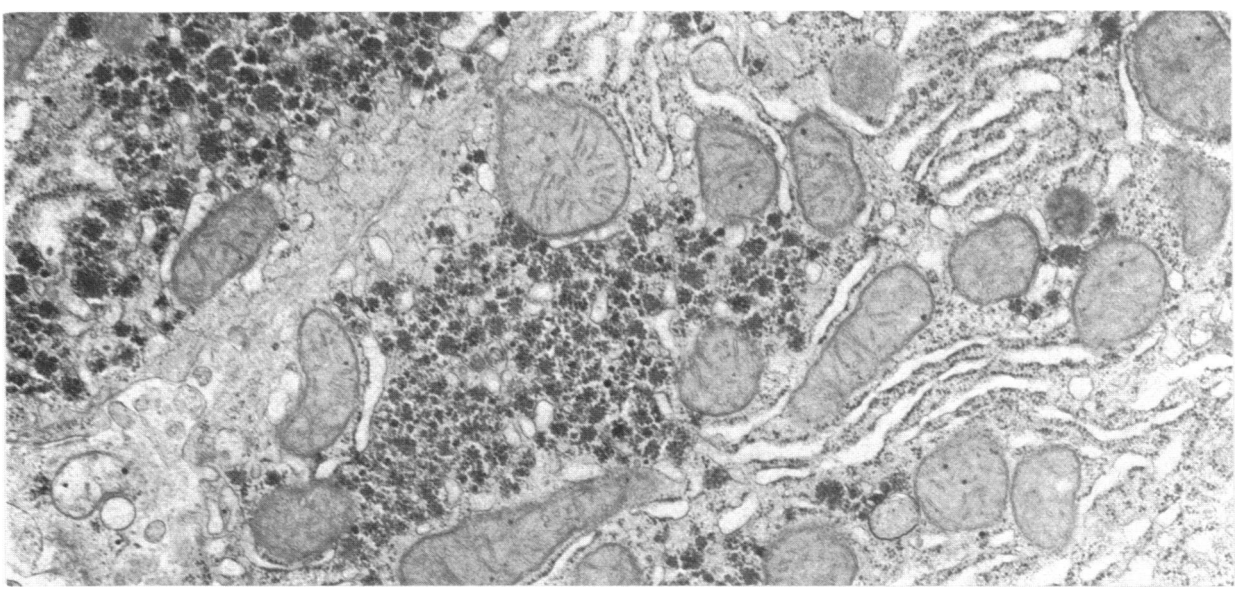

FIG. 2. Normal liver from a control rat showing abundant rough endoplasmic reticulum and smooth endoplasmic reticulum with multiple mitochondria interspersed. There are several masses of electron-dense glycogen in the cytoplasm. The glycogen particles are in both aggregated (α) and nonaggregated (β) forms. (Electron micrograph; ×17,400.)

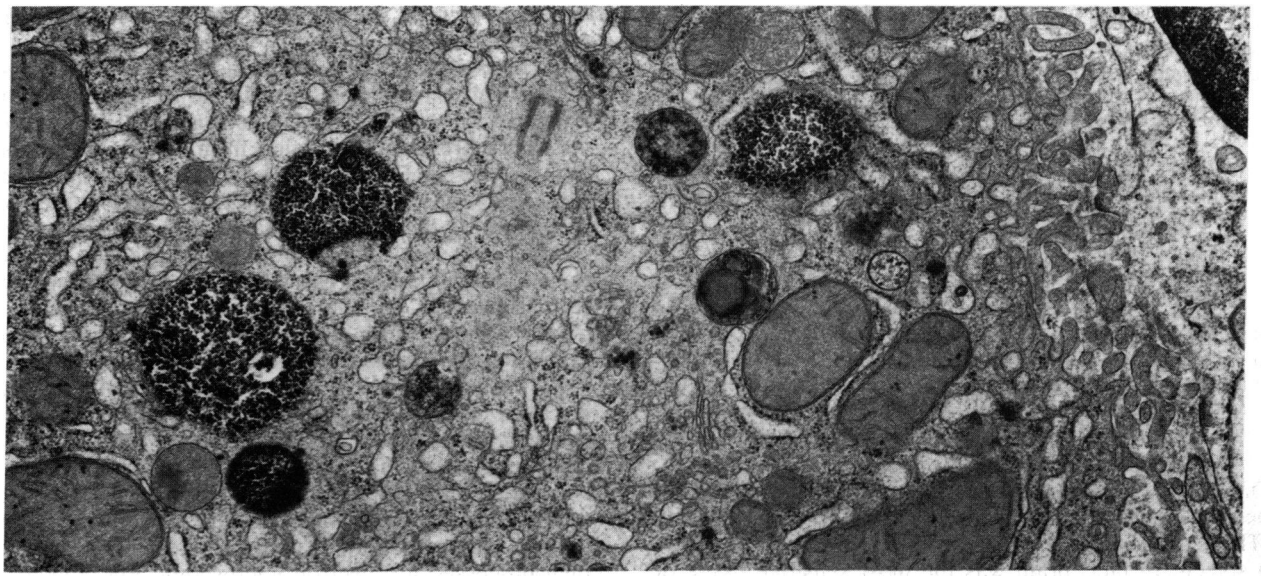

FIG. 3. Liver from an animal treated with castanospermine (2 mg/g of body weight) for 3 days and the specimen obtained on the fourth day. Cytoplasmic glycogen, rough endoplasmic reticulum, and smooth endoplasmic reticulum are markedly attenuated. Unique to the alkaloid-treated animals are numerous hepatic cytoplasmic vesicles filled with glycogen. These lysosomes measure as large as 3 μm in diameter and contain both α- and β-forms of glycogen. (Electron micrograph; ×17,400.)

measurement would probably not reveal small changes. Also, measurements of particle size in the electron micrographs did not indicate any substantial differences in size between treated and control animals. It is not clear why there is diminished cytoplasmic glycogen as well as lower total amounts of glycogen in treated animals, but it is possible that this alkaloid somehow interferes with glycogen synthesis. However, preliminary experiments indicate that castanospermine does not affect the *in vitro* assay of glycogen synthase or glycogen phosphorylase.

The results presented here indicate that castanospermine is an *in vivo* inhibitor of α-glucosidase when injected intraperitoneally into rats. Whether this treatment results in an actual decrease in the amount of enzyme or only affects the enzymatic activity remains to be established. Animal cells contain several α-glucosidase activities, some of which have acidic pH optima, while others have neutral pH optima. These hepatic enzymes with acidic optima are thought to be lysosomal, and at least one of these α-glucosidases has been purified to homogeneity and shown to have α-1,4- and α-1,6-glucosidase activity (8). This enzyme is missing or defective in individuals with Pompe disease, resulting in an abnormal accumulation of glycogen. In these patients, the glycogen abnormalities that occur in lysosomes of heart and skeletal muscle are associated with clinical manifestations of organ malfunction. Glycogen also accumulates in the lysosomes of liver (9, 10), as it did in the experimental animals described in this study.

A previous study with another α-glucosidase inhibitor, acarbose (11), gave results analogous to those described here. Rats given intraperitoneal injections of acarbose over a 7-day period showed intralysosomal glycogen storage within hepatocytes and Kupffer cells. The authors concluded that the cytologic picture resembled that of type II glycogenosis.

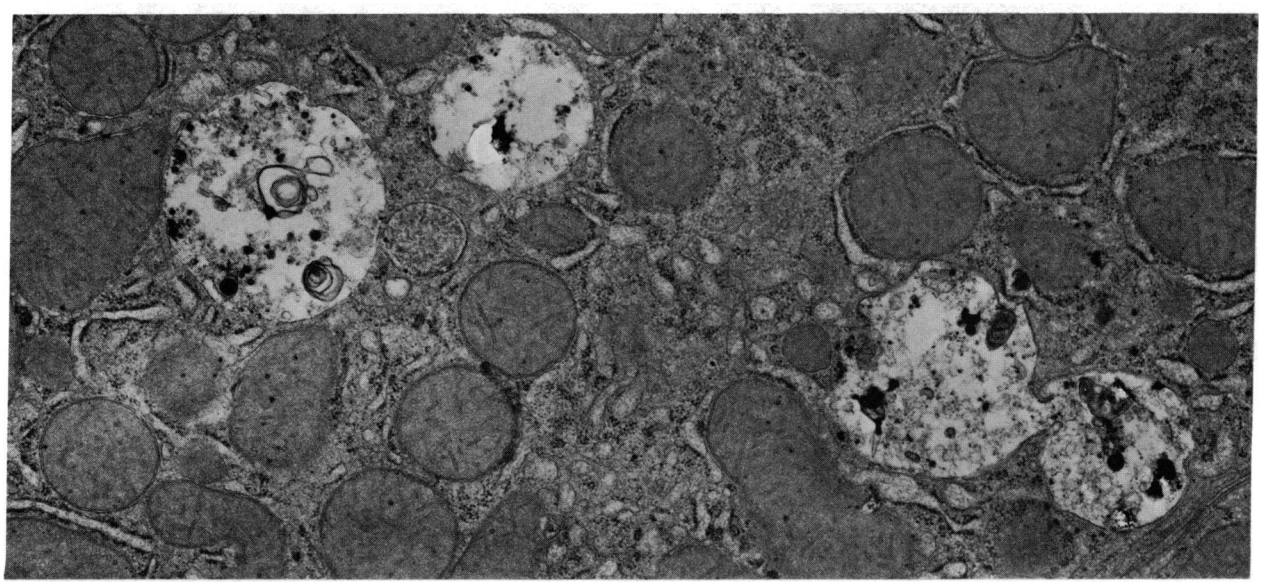

FIG. 4. Liver from a castanospermine-treated animal after the tissue blocks were subjected to α-amylase digestion. The general as well as vesicular (lysosomal) glycogen has been lysed. (Electron micrograph; ×17,400.)

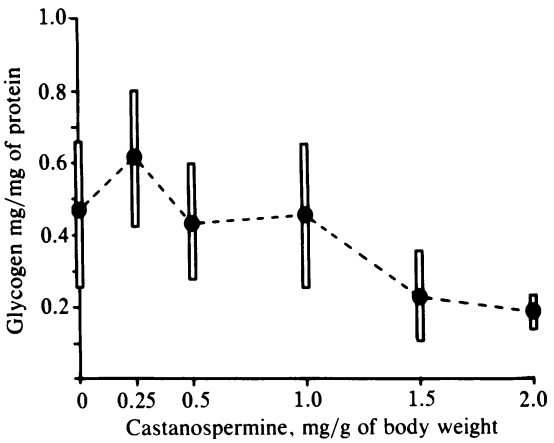

FIG. 5. Glycogen content of livers from control and experimental animals. Each point represents the mean of the glycogen levels found in seven separate animals given the same dose of castanospermine. The standard deviation of the mean is represented by the open bars.

Intralysosomal glycogen storage was also prominent in kidney, adrenal cortex, and spleen (12). Removal of the inhibitor resulted in a slow recovery. However, in studies using acarbose, no enzymatic activities were measured, and no correlations between glycogen levels or distribution and α-glucosidase activity could be drawn. It may be of interest that individuals with type II glycogenosis frequently have an elevation in the activity of β-*N*-acetylhexosaminidase of liver (10). In livers of animals treated with castanospermine, this enzymatic activity was also elevated.

Unpublished preliminary studies in our laboratory suggest that castanospermine also inhibits intestinal glycosidases (i.e., maltase and sucrase). Since the normal rat diet contains large amounts of sucrose and starch, and these sugars presumably cannot be metabolized in treated animals, the animals exhibit symptoms like those of individuals with lactose intolerance (i.e., high intestinal bacterial flora, diarrhea, etc.). Thus, the animals could be nutritionally deficient. However, this problem appeared to be overcome in the present studies by placing these animals on a different diet in which sucrose and starch were replaced with glucose. Such animals, even when treated with alkaloid, gained weight like the control animals, and the electron microscopy of the livers indicated that the hepatocytes were similar to those of controls with one exception. Glycogen was still found in lysosomes, which indicated that glucosidase activities in these animals were still inhibited. These findings were in complete agreement with our biochemical data showing a significant reduction in hepatic α-glucosidase activities. These studies indicate that castanospermine should be an interesting compound to use for studies on glycogen metabolism. It may also be of considerable value for dietary studies associated with sugar intolerance.

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