Glucuronide Metabolism in Plants

3. TRITERPENE GLUCURONIDES*

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The action of the enzyme β-glucuronidase on simple plant glucuronides in which the aglycone is flavonoid in nature has been dealt with in previous papers in this series (Levvy, 1954; Marsh, 1955*a*, *b*). Two triterpene glycosides containing glucuronic acid are known to occur in plants, and these have now been studied as substrates for β-glucuronidase.

Glycyrrhizinic acid, much the better-characterized of these triterpene glycosides, is present in large amounts in the root of liquorice (*Glycyrrhiza glabra*) and also in the commercial extract. There are numerous references to the occurrence of this, or a closely related, compound in many other plant genera (Czapek, 1925). It is a glucuronido-glucuronide of glycyrrhetic acid, a triterpene of the β-amyrin series (Ruzicka, Jeger & Winter, 1943), and the sugar residue has been shown by Lythgoe & Trippett (1950) to be a β-1′:2-diglucuronic acid. Considerable interest in glycyrrhizinic acid has recently been aroused by the pharmacological properties of the aglycone, which in some ways resemble those of deoxycorticosterone (Card *et al.* 1953).

A glycoside of oleanolic acid, a triterpene closely related to glycyrrhetic acid, has long been reported to be the chief constituent of sugar-beet 'saponin'. Glucuronic acid was the only sugar known to be present in the glycoside, which was considered to be a monoglucuronide (Rehorst, 1929). We have obtained this glycoside from fodder beet, as well as from the waste from beet-sugar manufacture. Farm animals may ingest beet saponin from more than one source, since by-products from the refining of beet sugar are employed in the manufacture of cattle cake.

EXPERIMENTAL

Methods

Enzyme preparation and assay. The preparation of mouse-liver β-glucuronidase and the measurement of its activity were carried out as described by Levvy & Marsh (1952). In the estimation of glucuronic acid as reducing sugar by the method of Levvy (1946), protein was precipitated with trichloroacetic acid instead of copper tungstate (cf. Levvy, 1954). Ammonium glycyrrhizinate, beet saponin and their aglycones did not interfere in these estimations.

Analyses. Chromatograms were prepared on Whatman no. 1 paper in all-glass tanks at room temp., by the descending method. Measurement of NH_3 was carried out with Nessler reagent (Snell & Snell, 1949), A.R. $(NH_4)_2SO_4$ being used as standard. Determinations of C, H and N were made by Weiler and Strauss, Oxford. Spectral absorption between 200 and 350 mμ. was measured with the Hilger Uvispek quartz spectrophotometer. All melting points are corrected.

Materials

Ammonium glycyrrhizinate. The procedure was based on those of Tschirsch & Cederberg (1907), Voss, Klein & Sauer (1937) and Lythgoe & Trippett (1950). Commercial 'glycyrrhizinum ammoniacale' (905 g.) was extracted with 2·2 l. of boiling acetic acid, and after cooling to about 50° the solution was separated from insoluble material on the centrifuge. The solid which was deposited after 2 weeks at room temp. was thrice recrystallized from acetic acid, washed with acetic acid and then with ethanol, and dried over KOH. The yield was 74 g. of crude ammonium glycyrrhizinate, and 40 g. of this was dissolved in warm 20% (v/v) aqueous ethanol (800 ml.), made alkaline to litmus with conc. NH_3 soln. and filtered. Excess of basic lead acetate was added, and the precipitated lead salt was washed with 20% ethanol, suspended in 2 l. of 30% (v/v) aqueous ethanol, and decomposed with H_2S. Lead sulphide was removed by centrifuging and the supernatant was evaporated to dryness *in vacuo*. The residue was recrystallized, with hot filtration, from 40% and then from 75% (v/v) aqueous ethanol to give 10·4 g. of pure ammonium glycyrrhizinate pentahydrate as colourless needles; m.p. 212–217° (decomp.) and $[\alpha]_D^{20}$ +46·9° in 40% ethanol (*c*, 1·5). Lythgoe & Trippett (1950) give $[\alpha]_D^{17}$ +43·2°. (Found: loss at 80° *in vacuo* 9·7%; equiv. by titration to pH 7, 477. Calc. for $C_{42}H_{65}O_{16}N,5H_2O$: H_2O, 9·7%; equiv., 465. Found for anhydrous product: C, 59·4; H, 8·1; N, 1·68; NH_3, 1·98. Calc. for $C_{42}H_{65}O_{16}N$: C, 60·0; H, 7·8; N, 1·67; NH_3, 2·03%.) There was a single absorption maximum at 248 mμ. (0·1 mM in ethanol); $\epsilon_{max.}$ 11 400.

Glycyrrhetic acid. Ammonium glycyrrhizinate pentahydrate (0·74 g.) was heated at 100° under N_2 with N-H_2SO_4 (55 ml.) for 5 hr. The glycyrrhetic acid (0·35 g., 90% of theory) was filtered off and recrystallized from 75% (v/v) aqueous ethanol, m.p. 284–285°. Voss *et al.* (1937) give m.p. 283° for α-glycyrrhetic acid. Absorption was almost identical with that of ammonium glycyrrhizinate, with a single maximum at 249 mμ. (0·1 mM in ethanol), $\epsilon_{max.}$ 12 100. Nieman (1952) found optimum absorption at 250 mμ.

* Part 2: Marsh (1955*a*).

Saponin from sugar-beet waste. Preliminary experiments showed that this compound was present in various waste products from sugar refining supplied by the British Sugar Corporation, Cupar, Fife. The spent cake from the liming process was the most convenient source.

Moist spent cake (2 kg., 840 g. dry weight) was suspended in water (4 l.), and mechanically stirred. Conc. HCl (about 1·1 l.) was slowly added until effervescence had ceased and the pH of the liquid was 1·5–2. The mixture was centrifuged and the supernatant was discarded. After washing with 0·05 N-HCl (800 ml.), the grey sediment was resuspended in water (1 l.) and 3 N-NaOH was stirred in until the pH was 9. Alkali-insoluble material was separated by centrifuging and washed with 0·01 N-NaOH and then with water. The combined supernatant and washings were brought to pH 2 with 3 N-HCl, and the precipitate which separated overnight at room temp. was washed with 0·05 N-HCl and dried *in vacuo* at 80° over KOH. A total yield of 750 g. was obtained from 13 kg. of moist lime cake.

The powdered crude product was worked up in batches: 100 g. was extracted twice with boiling methanol (total 800 ml.), and the extract was evaporated to dryness. Since the residue contained nitrogen, it was suspended in water (500 ml.) and brought to pH 9 with NaOH. After maintaining the suspension at 80° for 15 min., it was cooled and centrifuged, and the sediment was discarded. The N-free glycoside was then precipitated with HCl. The combined batches gave a total product of 62 g. To remove less-soluble, non-uronide impurities, this was dissolved in methanol (600 ml.) and ether (3 l.) was added. After filtering, removal of the solvent yielded a yellow solid (56 g.), which still contained the more soluble impurity, free oleanolic acid. This was removed by extraction with cold ether (500 ml.), leaving 31 g. of residue, m.p. 205–209° (decomp.). Recrystallization of the glycoside from ethyl acetate gave a white powder, m.p. 217–219° (decomp.). Eis, Clark, McGinnis & Alston (1952) quote m.p. 214–216° (decomp.) for sugar-beet saponin. After drying at 80° *in vacuo* over P_2O_5, the loss in weight was 8·9 %. [Found for anhydrous product: C, 62·7; H, 8·1. Calc. for $C_{42}H_{66}O_{14}$ (see below): C, 63·5; H, 8·4 %.]

Saponin from fodder-beet. Freshly cut fodder-beet (var. Red Otofte), from the Duthie Farm attached to this Institute, was used. Only the epidermal layer yielded appreciable amounts of acid-precipitable material (cf. Grebinskiĭ & Lenkova, 1949, 1950). Finely grated peel (500 g. from 3 kg. of whole beet) was suspended in 2 l. of 0·1 N-H_3PO_4–NaOH buffer, pH 7, and stirred for 2 hr. at 100°. The liquor was strained through muslin, concentrated *in vacuo* to 500 ml. and centrifuged. After the clear supernatant had been brought to pH 2 with conc. HCl, it was set aside at 0° overnight. The precipitate which formed was separated by centrifuging and washed with 0·05 N-HCl, and the glycoside was extracted with NaOH and reprecipitated with HCl, to yield a brown, amorphous, N-free product (2·0 g.). This was dissolved in hot methanol, and the solution was filtered. After removal of methanol, the residue was extracted with cold ether to remove oleanolic acid. Recrystallization of the ether-insoluble residue from ethyl acetate, with hot filtration, gave a white powder, m.p. 214–216° (decomp.), not depressed by admixture with the product from sugar-beet waste. The material insoluble in ethyl acetate gave a strong Tollens colour reaction, and left an appreciable residue on combustion. It thus resembled the glycoside–salt complex from sugar-beet described by Eis *et al.* (1952).

The pure products from sugar- and fodder-beet gave strong Tollens colour reactions for uronic acid, and reduced Fehling's solution only after hydrolysis with 2 N-HCl at 100° for 15 min.

Oleanolic acid. A solution of beet saponin (1·0 g.) in ethanol (10 ml.) was added to 4 N-HCl (10 ml.), and the mixture was refluxed for 4 hr. The precipitate (0·42 g.) was extracted with cold ether (20 ml.). Crude oleanolic acid (0·38 g.) from the ether extract was thrice recrystallized from 80 % (v/v) aqueous ethanol and finally from absolute ethanol; the product had m.p. 304–306°, $[\alpha]_D^{15}$ +76° in $CHCl_3$ (*c*, 1). M.p. and mixed m.p. of products from sugar- and fodder-beet were identical. According to van der Haar (1927), oleanolic acid from sugar-beet has m.p. 307–308° and $[\alpha]_D^{12}$ +79·5°.

Preparation of oleanolic acid monoacetate according to the method of Markley, Sandor & Hendricks (1938) gave products from sugar- and fodder-beet with m.p. and mixed m.p. 263–264°. Markley *et al.* (1938) quote m.p. 264–265°.

4-O-*Methyl*-D-*glucurone.* The amide of methyl 4-*O*-methyl-α-D-glucuronide prepared from mesquite gum (Smith, 1951) was warmed with dilute NaOH (Smith, 1951) to decompose the amide group. The solution was made N with respect to HCl and boiled for 5 hr. to split the glycoside link. Chloride ion was removed by shaking with Ag_2CO_3. The filtered solution was treated with Amberlite IR-120 (H) to remove cation, and taken to dryness *in vacuo* to yield a yellow gum, which behaved titrimetrically (Marsh, 1955*c*) and chromatographically (see Fig. 2) as a lactone. Neither the starting material nor the final product gave the Tollens colour reaction for uronic acids.

RESULTS

Hydrolysis by β-glucuronidase

The liberation of reducing sugar, calculated as glucuronic acid, from ammonium glycyrrhizinate and beet saponin in presence of mouse-liver β-glucuronidase is shown in Table 1. Parallel experiments were done with (−)-menthol α- and β-D-glucuronides: as previously noted (Levvy & Marsh, 1952), the α-anomer was not hydrolysed by the enzyme. β-Glucuronidase liberated reducing sugar from ammonium glycyrrhizinate, and this effect was suppressed by boiled saccharate solution (Levvy, 1952), a specific inhibitor for the enzyme.

Fig. 1 shows that prolonged incubation of ammonium glycyrrhizinate with the enzyme led to the liberation of two molecules of glucuronic acid from each molecule of glucuronide present. In a larger-scale experiment, 0·12 g. of ammonium glycyrrhizinate pentahydrate was incubated with the enzyme for 120 hr. at pH 5·2. At the end of this period, 79 % of the glucuronic acid was liberated as reducing sugar. The white precipitate which had formed was separated by centrifuging, washed with water and extracted with ethanol. Evaporation of the ethanolic extract yielded 0·048 g. of glycyrrhetic acid (corresponding to 76 % hydrolysis), which after recrystallization from 70 % (v/v) aqueous ethanol had m.p. 287–289°. There was no depres-

Table 1. *Liberation of reducing sugar from ammonium glycyrrhizinate and beet saponin by mouse-liver β-glucuronidase*

Hydrolysis mixture (0·8 ml.) incubated for 2 hr. at 37° and pH 5·2 in sodium acetate–acetic acid buffer, final concentration 0·125 N. Saccharate solution boiled for 30 min. before use.

Substrate	Concn. (mM)	Inhibitor	Glucuronic acid liberated (μg.)	Hydrolysis (%)	Inhibition (%)
(−)-Menthol α-D-glucuronide	5	None	0	0	—
(−)-Menthol β-D-glucuronide	10	None	127*	8	—
(−)-Menthol β-D-glucuronide	10	Saccharate (0·025 mM)	15*	1	88
Ammonium glycyrrhizinate	1	None	79	13†	—
Ammonium glycyrrhizinate	1	Saccharate (0·025 mM)	5	1†	94
Beet saponin	0·08%	None	0	0	—

* Enzyme preparation diluted 1:1. † Calculated on diglucuronide.

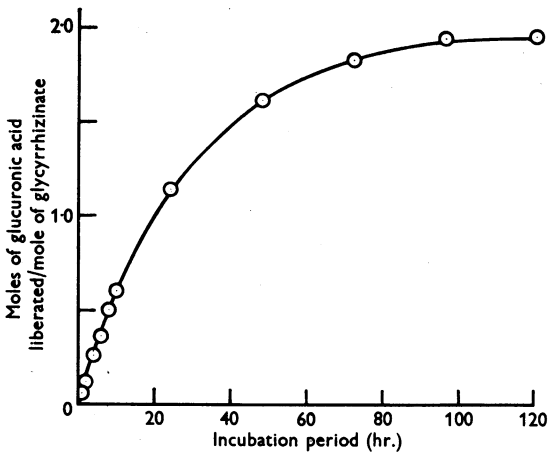

Fig. 1. Liberation of glucuronic acid from mM ammonium glycyrrhizinate by mouse-liver β-glucuronidase after varying periods at 37° and pH 5·2.

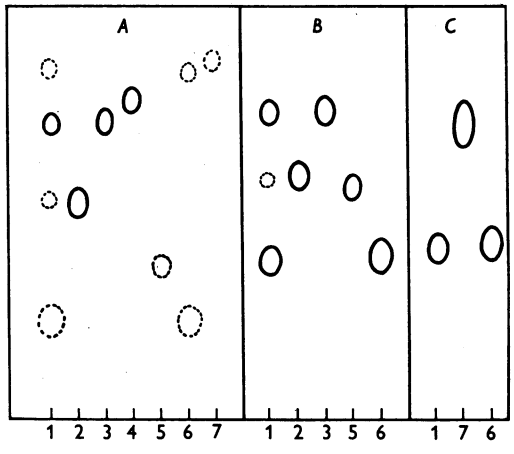

Fig. 2. Paper chromatography of the sugars present in acid hydrolysates of beet saponin. Chromatograms run for 46 hr. Spots: 1, beet saponin; 2, xylose; 3, glucose; 4, galactose; 5, 4-methylglucurone; 6, glucurone; 7, galacturonic acid. *A*, Solvent, *n*-butanol–pyridine–water; spray, aniline phosphate in butanol. *B*, Solvent, *n*-butanol–acetic acid–water; spray, benzidine–trichloroacetic acid. *C*, Solvent, *n*-butanol–acetic acid–water; spray, naphthoresorcinol in HCl. Faint spots are indicated by broken lines.

sion of the m.p. on admixture with the aglycone prepared by acid hydrolysis. It is evident that both glucuronide residues in glycyrrhizinic acid have the β-configuration.

Unlike ammonium glycyrrhizinate, the beet saponin never showed any liberation of reducing sugar on incubation with β-glucuronidase (Table 1).

Chromatographic analysis of the sugar residue in beet saponin

β-Glucuronidase has been shown to be specific for β-glucopyranuronides (Levvy & Marsh, 1952). If beet saponin is an α-glucuronide or a glucofuranuronide, it should not be hydrolysed by the enzyme. The failure to observe hydrolysis might, however, also be due to the presence of a substituent in the glucuronide residue. This possibility was examined by paper chromatography.

The acid hydrolysate from the preparation of oleanolic acid (see above) was filtered and evaporated *in vacuo* to give a brown gum. This was dissolved in water and shaken with Ag_2CO_3 to remove chloride ion. After filtration through charcoal, the solution was shaken with Amberlite IR-120 (H) for 1 hr. to remove cation. Evaporation of the resultant colourless solution *in vacuo* yielded a pale-yellow gum (0·29 g./g. of saponin), which was dissolved in water.

The chromatographic solvent was either the upper phase of a *n*-butanol–acetic acid–water

mixture (4:1:5, by vol.) or a *n*-butanol–pyridine–water mixture (10:3:3, by vol.). D-Glucurone gave only one spot (unchanged lactone) in the former solvent, and two (glucurone and glucuronic acid) in the latter. D-Galacturonic acid and 4-*O*-methyl-D-glucurone always gave single spots, corresponding to the free acid and unchanged lactone respectively. The chromatograms were sprayed with benzidine–trichloroacetic acid, aniline phosphate in *n*-butanol or naphthoresorcinol in 0·5N-HCl. All the reference compounds of Fig. 2 reacted with benzidine. The uronic acids gave only a faint reaction with aniline. Naphthoresorcinol was completely specific for the unsubstituted uronic acids or lactones. As shown in Fig. 2, the beet saponin hydrolysate contained glucuronic acid and a hexose, probably glucose, in approximately equal proportions. Traces of xylose were sometimes observed, derived no doubt from decarboxylation of glucuronic acid during the acid hydrolysis. The other reference compounds were absent from the hydrolysate.

The percentages of C and H in the saponin were in accordance with the theory for a compound ($C_{42}H_{66}O_{14}$) containing one molecule of glucose and one of glucuronic acid for each molecule of oleanolic acid (see above).

Inhibition of the hydrolysis of phenolphthalein glucuronide

As expected, ammonium glycyrrhizinate depressed the release of phenolphthalein from phenolphthalein glucuronide by mouse-liver β-glucuronidase. The effect depended on pH. In a concentration of 0·1 mM (about 0·01 %), glycyrrhizinate caused 51 % inhibition of the hydrolysis of 0·5 mM phenolphthalein glucuronide at pH 4·5, and 34 % inhibition at pH 5·2. Fig. 3 shows the effect of varying the substrate concentration at fixed inhibitor concentration. It can be seen that inhibition was in part competitive and in part non-competitive, since both K_m, the dissociation constant of the active enzyme–substrate complex, and V_m, the maximum rate of hydrolysis, were altered. Analysis of the results by the method of Lineweaver & Burk (1934), as modified by Dixon (1953), gave values at pH 5·2 of 0·185 and 0·21 mM for K_i, the dissociation constant of the enzyme–inhibitor complex, for the competitive and non-competitive components respectively. At pH 4·5, the corresponding values were 0·37 and 0·081 mM. Decreasing the pH increased the affinity ($1/K_i$) of the non-competitive component at the expense of the competitive one. As usual, excess of phenolphthalein glucuronide depressed the activity of the enzyme.

The competitive component in the inhibitory action of glycyrrhizinate can be explained by the fact that it is an alternative substrate for the enzyme. Since the inhibitor is a ββ′-diglucuronide, K_i (competitive) must itself be a composite value. In a concentration of mM, glycyrrhetic acid caused only small, variable inhibitory effects, never exceeding 6 %, in the hydrolysis of 0·63 mM phenolphthalein glucuronide. It would therefore appear that the non-competitive component in the

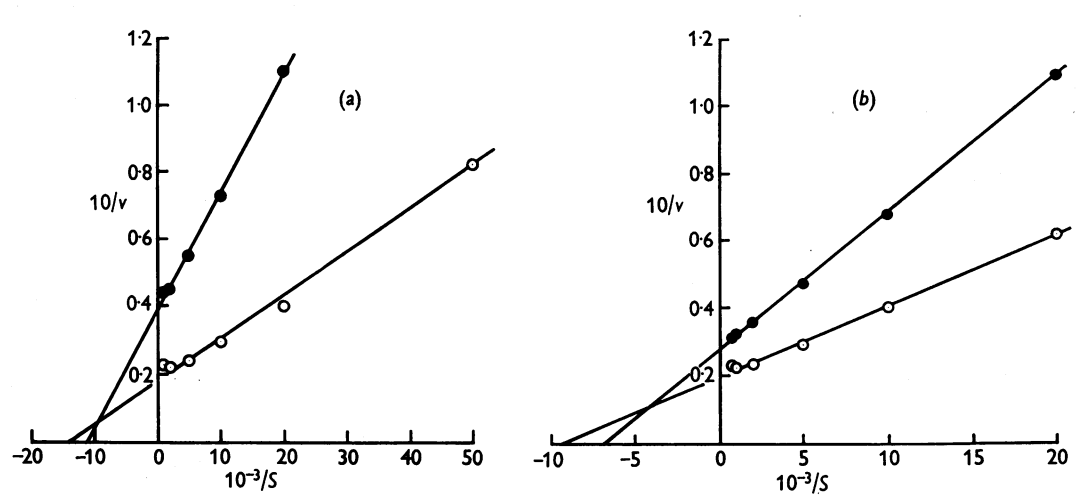

Fig. 3. Effect of varying the phenolphthalein glucuronide concentration (S) on the rate of release of phenolphthalein (v) by mouse-liver β-glucuronidase in presence (●) and absence (○) of 0·1 mM ammonium glycyrrhizinate. Incubation for 1 hr. at 38° and pH 4·5 (*a*) and 5·2 (*b*) in 0·125N sodium acetate–acetic acid buffer. A single enzyme preparation was used throughout. Results are plotted as $1/v$ against $1/S$ (Dixon, 1953). The intercept on the vertical axis is equal to $1/V_m$ and on the horizontal axis to $-1/K_m$ (see text).

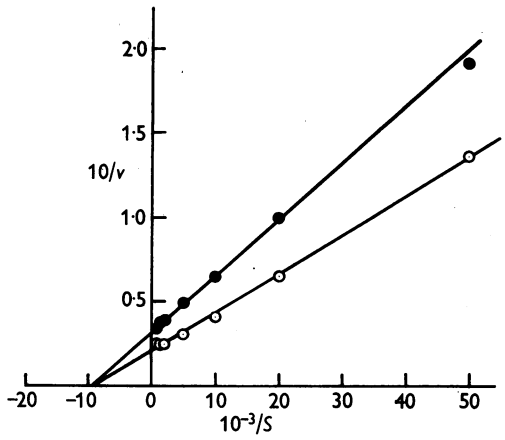

Fig. 4. Effect of varying the phenolphthalein glucuronide concentration (S) on the rate of release of phenolphthalein (v) by mouse-liver β-glucuronidase in the presence (●) and absence (○) of 0·04% (w/v) beet saponin at pH 5·2. Details as in Fig. 3.

inhibitory action of ammonium glycyrrhizinate was due to the intact glycoside or its half-hydrolysis product.

Beet saponin, which is not a substrate for the enzyme, inhibited the hydrolysis of phenolphthalein glucuronide by β-glucuronidase, and the effect was entirely non-competitive (Fig. 4; $K_i = 0{\cdot}084\%$). Oleanolic acid caused no inhibition.

DISCUSSION

The complete hydrolysis of glycyrrhizinic acid by β-glucuronidase proves that both of the glucuronic acid residues have the β-configuration. So far as the interglucuronide link is concerned, this is in agreement with the conclusions arrived at by previous workers from degradation studies (Voss & Pfirschke, 1937; Lythgoe & Trippett, 1950). Lythgoe & Trippett (1950) carried out partial acid methanolysis of fully methylated glycyrrhizinic acid. From the molecular rotation of the methylated methyl diglucuronide thus obtained, they calculated that the methyl–glycoside link had the α-configuration. From this they reasoned that the bond between the aglycone and the disaccharide residue in glycyrrhizinic acid also had the α-configuration. In general, however, acid methanolysis of a glycoside bond, irrespective of its configuration, results in an equilibrium mixture of α- and β-methyl glycosides, in which the α-anomer may predominate (e.g. see Smith, 1951).

Three possible explanations of the fact that, in spite of the presence of a glucuronide residue, beet saponin was not hydrolysed by β-glucuronidase are: (*a*) the glucuronide residue has the α-configuration; (*b*) it is in the furanose form; (*c*) the presence of a substituent in the glucuronide residue prevents hydrolysis of a β-glucopyranuronide link by the enzyme. Levvy & Marsh (1952) obtained some evidence for the failure of β-glucuronidase to act on an *O*-substituted glucuronide (cf. Linker, Meyer & Weissmann, 1955). Chromatographic evidence for the presence of a second sugar, probably glucose, in the saponin molecule is consistent with the third possibility. There being only one hydroxyl group in oleanolic acid, the two sugar residues in beet saponin must be linked together, although there is no evidence for the structure of the disaccharide residue. A fourth possibility, that the glucuronic acid residue in beet saponin is in the form of the lactone, can be discounted, since the saponin titrated entirely as a free acid.

Although it was not a substrate for β-glucuronidase, beet saponin resembled glycyrrhizinic acid to the extent of causing non-competitive inhibition of the enzyme. Inhibition of this enzyme is not, it should be noted, a general property of triterpene saponins (cf. Rossiter & Wong, 1950).

SUMMARY

1. Both the glucuronide residues in ammonium glycyrrhizinate were removed by hydrolysis with β-glucuronidase, proving that this glucuronido-glucuronide has the ββ′-configuration.

2. Although it contains a glucuronide residue, beet saponin was not hydrolysed by β-glucuronidase. Chromatographic analysis of an acid hydrolysate revealed the presence of a second sugar, probably glucose, in this glycoside. The possible structure of the glycoside group is discussed.

3. Beet saponin and ammonium glycyrrhizinate acted as non-competitive inhibitors in the hydrolysis of phenolphthalein glucuronide by β-glucuronidase. In addition, the glycyrrhizinate behaved, as expected, as a competing substrate for the enzyme.

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Studies in the Reaction of Amino and Imino Compounds with Sugars: the Reaction of Histidine with Glucose

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The reaction of amino acids, peptides and proteins with various sugars has been extensively investigated and reviewed (Danhey & Pigman, 1951), but the results obtained have been more often than not contradictory. These discrepancies may be due to various causes, most prominent being the failure to obtain equilibrium conditions and the likelihood that different types of reaction take place at different concentrations, pH values and temperatures. The effect of concentration of glucose or amino acid on the equilibrium constant and the degree of combination at different pH values was studied at a single temperature by Katchalsky (1941) and by Frankel & Katchalsky (1941). They used a potentiometric technique similar to that employed by Levy (1933) and by Balson & Lawson (1936), who investigated the combination of amino acids with formaldehyde. The procedure involved measurements of the decrease in pH brought about by the combination. Katchalsky (1941) interpreted his results in terms of combination between one molecule of the anionic form of the amino compound and one molecule of glucose, and he calculated the corresponding equilibrium constants for the one temperature (23°) employed. He also stated that the values of the equilibrium constants were generally independent of the concentration of both components and of pH over the range 7–9. His method of varying the concentrations was to mix different quantities of the two stock solutions (i.e. the amino acid solution adjusted to a certain pH and the glucose solution), and to keep the total volume constant. However, such a procedure cannot be considered ideal, since increase in the concentration of one component is accompanied simultaneously by decrease in concentration of the second component, and this may result in masking of opposing effects on the equilibrium constant. The fundamental work of Frankel & Katchalsky (1941) left unsolved the problem whether the reaction between amino compounds and sugars was of the same type at other temperatures. Several investigators, including Borsook & Wasteneys (1925), Euler, Brunius & Josephson (1926*a*, *b*) and Lieben & Getreuer (1932), observed that amino acid–glucose mixtures were capable of reducing methylene blue at 37° and higher temperatures. The present author has confirmed most of these observations and examined the kinetics of the glycine–glucose reduction of several indicators (unpublished work). This oxidation–reduction reaction is of physiological interest, since its mechanism may parallel some of the steps involved in the enzymic dehydrogenation of glucose. The 'browning reaction' in amino acid–sugar mixtures indicates that amino compounds and glucose may combine or react in more than one way, and it is therefore possible that the reaction