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Transglucosidation Reactions with Flavins

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The enzymic formation of riboflavinyl glucoside by a transfer of glucose from maltose to riboflavin was described by Whitby (1952), and the reaction may be written thus:

It was concluded that a transglucosidase catalysed this reaction, and the characteristics of this enzyme, particularly with regard to its specificity, have now been investigated in an attempt to assess the physiological significance of riboflavinyl glucoside.

EXPERIMENTAL

Materials

D-Riboflavin (Roche Products, Ltd.) was used, and riboflavinyl glucoside was prepared enzymically (Whitby, 1952). D- and L-Arabinoflavin and the 9-substituted-isoalloxazines used in this work were the generous gift of Prof. P. Karrer; galactoflavin, L-lyxoflavin and isoriboflavin were kindly supplied by Dr M. Tishler. Flavin solutions were at all times protected from light as much as possible.

6:7-Dimethyl-9-(5'-[β -D-glucopyranosyl]-D-ribityl)-isoalloxazine and 6:7-dimethyl-9-(4'-[α -D-glucopyranosyl]-D-glucityl)-isoalloxazine have been synthesized (Plant, 1952); small amounts of these two compounds, riboflavinyl β -D-glucoside and maltoflavin, respectively, were kindly provided by Dr Plant.

α -D-Glucose 1-phosphate (dipotassium salt) was prepared by the method of Hanes (1940).

Methyl maltoside was prepared by refluxing maltose with methanolic HCl (Fischer, 1893, 1895). Other carbohydrates and derivatives were commercial samples, except for melezitose and turanose, kindly supplied by Dr D. J. Bell, and isomaltose octaacetate, maltulose and panose which were kindly provided by Dr W. J. Whelan.

isoMaltose octaacetate (15 mg.) was saponified with sodium methoxide under conditions similar to those used by Wolfrom, Georges & Miller (1949); the methanolic solution was diluted with water at 0°, immediately neutralized with HCl, and the methanol removed *in vacuo* at 35°. The solid

residue was dissolved in water and the solution used for enzymic tests; it showed only one spot on chromatograms which had been developed with butanol:acetic acid:water (4:1:5, v/v/v) and sprayed with aniline hydrogen phthalate (Partridge, 1949), the spot having $R_F = 0.10$, compared with 0.12 for maltose.

Phosphate buffers were prepared with K_2HPO_4 and $NaH_2PO_4 \cdot 2H_2O$.

Enzyme preparation. An acetone powder of rat liver was made by the method of Whitby (1952), and the following manipulations were all carried out in a cold room at 2°. The powder (40 g.) was extracted for 20 min. in a mortar with 400 ml. of 0.1 M phosphate buffer, pH 7.4, and the suspension centrifuged for 15 min. at 1800 g; the supernatant was collected, the centrifuge extracted a second time with 400 ml. of buffer for 20 min., centrifuged, the supernatants combined (728 ml.) and the solid residue discarded. M-Phosphate buffer (182 ml.), pH 7.4, and 131 g. of $(NH_4)_2SO_4$ were added. After 30 min. the precipitate was collected by centrifuging at 1800 g for 10 min. and was discarded. $(NH_4)_2SO_4$ was added to increase the saturation from 0.25 to 0.55 and the precipitate was collected 1 hr. later by centrifuging for 5 min. at 15000 g (Servall Centrifuge); the supernatant was discarded. The precipitate was dissolved in 50 ml. of 0.05 M phosphate buffer, pH 7.4, and dialysed overnight with stirring against 4 l. of 0.05 M phosphate buffer, pH 7.4. The dialysed solution was frozen at -20° and dried *in vacuo* over H_2SO_4 and NaOH. The powder weighed 7.3 g. and is henceforth referred to as 'the enzyme'; its activity did not deteriorate over 6 months when stored *in vacuo* over $CaCl_2$. Attempts at further purification of the enzyme by preferential thermal inactivation of contaminating protein, with and without the addition of maltose to protect the enzyme, by adsorption and elution from calcium phosphate gel, by precipitation below pH 5.5, etc., have not been successful; however, this impure enzyme has proved to be satisfactory for the following experiments.

Methods

The pH was measured with a Cambridge pH meter (Cambridge Instrument Co.). The instrument was equilibrated at 35° for 2 days before determining the pH/activity relationship for synthesis of riboflavinyl glucoside.

Spectrophotometric measurements were made in quartz cells, 1 cm. thickness, in a Beckman model DU spectrophotometer. Flavins were estimated in 0.1M phosphate buffer, pH 7.4. Solutions of all the 6:7-dimethyl-9-substituted-isoalloxazines investigated here, e.g. riboflavin and galactoflavin, were estimated by their light absorption at 450 m μ ., using the molecular extinction coefficient (ϵ) = 12.2×10^3 l. mole⁻¹ cm.⁻¹ (Singer & Kearney, 1950a; Whitby, 1952), because of the close similarity of their absorption spectra. isoRiboflavin was estimated at 450 m μ ., using $\epsilon = 7.3 \times 10^3$ l. mole⁻¹ cm.⁻¹ (Tishler, Pfister, Babson, Ladenburg & Fleming, 1947). The three 9-substituted-isoalloxazines had similar spectra, with a maximum at 435 m μ ., which was used for their estimation. The value of ϵ at 435 m μ . was determined with a sample of 9-(L-1'-arabityl)-isoalloxazine which had been dried *in vacuo* at 100° over P₂O₅ for 1 hr., and $\epsilon = 10.4 \times 10^3$ l. mole⁻¹ cm.⁻¹ was observed. (Found: C, 50.7; H, 4.6; N, 20.1. Calc. for C₁₅H₁₆O₆N₄: C, 51.7; H, 5.2; N, 20.1 %.) Kuhn & Weygand (1934) observed that 9-methyl-isoalloxazine had an absorption maximum at 440 m μ ., and from their spectrum $\epsilon \approx 9.4 \times 10^3$ l. mole⁻¹ cm.⁻¹ at this wavelength.

Fluorimetric measurements were made with a fluorimetric attachment supplied for use with Beckman spectrophotometers. Measurements were made in 0.1M phosphate buffer, pH 7.4, and standard curves were obtained with riboflavin and with 9-(L-1'-arabityl)-isoalloxazine for estimating the glucosides of 6:7-dimethyl-9-substituted-isoalloxazines (e.g. riboflavinyl glucoside) and of 9-substituted-isoalloxazines, respectively; a separate standard curve was made with isoRiboflavin. The curves were linear up to a concentration of 1 μ g. of flavin/ml., and the fluorescence of riboflavinyl glucoside was found to be quantitatively equivalent to that of riboflavin under these conditions.

Estimation of enzymic activity. The contents of each incubation mixture are detailed below, but the preparation of the chromatograms and the estimation of flavin glucoside were performed similarly in each case. The estimation of the Michaelis constant for riboflavin in the synthesis of riboflavinyl glucoside from riboflavin and maltose is described here to illustrate the procedure.

Enzyme (160 mg.) was dissolved in 4 ml. of 0.1M maltose and 4 ml. of M phosphate buffer, pH 7.4, and kept at 0°; 1 ml. of this solution was added to tubes containing 0.5 ml. of riboflavin solution of different strengths (prepared by dilution of a supersaturated (1.94 mM) solution of riboflavin) and the solutions incubated in darkness at 35°. Sheets of Whatman no. 3 filter paper (57 $\times$ 46 cm.) had been prepared, and 0.1 ml. samples of each incubation mixture were applied along a line ruled parallel to the longer side of the paper, 8 cm. distant from its edge. Samples were applied with the minimum exposure to direct light and, to minimize the area of each spot as well as to reduce the time needed for application, the paper was heated during the application of a sample by pressing against glass tubing through which water was syphoning from a bath of boiling water. The spots were approximately 4 cm. wide and 1.5 cm. deep and the centres of spots were 8 cm. apart; samples were taken in triplicate at 0, 1, 2 and 3 hr. (except where otherwise stated) and, immediately after the application of samples at each of these times, the papers were heated in an oven at 105° for 5 min. to denature the enzyme. The papers were equilibrated with solvent vapour (water, saturated with isoamyl alcohol) for 6 hr. and then run as ascending chromatograms for 12 hr.

After development, chromatograms were dried in darkness at 37°; it was essential to hang the chromatograms at right-angles to the direction of solvent development, as otherwise the separation of flavin glucoside from unchanged flavin was sometimes impaired by migration of spots during the drying process. The chromatograms were examined by ultraviolet light and the areas containing flavin glucoside marked out with pencil. Riboflavinyl glucoside has $R_F = 0.5$ and riboflavin $R_F = 0.4$ in this solvent; other flavin glucosides are separated similarly from their parent flavins. Each spot was cut out and extracted with 4 ml. of 0.1M phosphate buffer, pH 7.4, by heating in glass-stoppered tubes for 10 min. in darkness at 60–70°. The solutions were cooled and most of the filter paper was removed with a forceps; fine particles of filter paper were removed by centrifuging, and the flavin glucoside was then estimated in the fluorimeter. Recovery of standard riboflavin solutions, applied to filter paper and eluted as described above, was 95–98%. Excessive exposure to light during the early manipulations led to the appearance of lumichrome, a blue-fluorescent spot having $R_F = 0.1$, on the chromatograms.

In experiments on flavin specificity, where sufficient flavin was added to saturate the solutions, samples of each incubation mixture were cooled rapidly to 0° and centrifuged at 0° before being applied to filter paper. This avoided the excessive trailing which occurs whenever undissolved flavins are applied to chromatograms.

In the experiments on carbohydrate specificity, a control determination was made with riboflavin present, but with no maltose nor other carbohydrate added, since the enzyme preparation contained a small amount of glucose donor from which riboflavinyl glucoside was synthesized in minute amount, and a correction could thus be applied.

RESULTS

Quantitative characteristics of the transglucosidase

Riboflavin. The experimental details are described in the previous section, and the results were plotted by the method of Lineweaver & Burk (1934). The Michaelis constant for riboflavin at 35° and pH 7.4 in reaction (1) was found to be 0.41 mM.

Maltose. 0.05M Maltose was prepared in a supersaturated solution of riboflavin (2.75 mM in 0.5M phosphate buffer, pH 7.4) and a series of dilutions of this maltose solution made by dilution with the riboflavin solution. The enzyme was also dissolved in more of this supersaturated solution of riboflavin in order that the Michaelis constant for maltose might be determined in the presence of a high concentration of riboflavin. The Michaelis constant for maltose at 35° and pH 7.4 in reaction (1) was found to be 0.82 mM.

α -D-Glucose 1-phosphate. The previous experiment was repeated in the presence of α -glucose 1-phosphate (1.50 mM) and the results are shown in Fig. 1. The inhibition of reaction (1) by α -glucose 1-phosphate is competitive, and the inhibitor constant at 35° and pH 7.4 is 1.7 mM.

pH/activity relationship was investigated between pH 5.7 and 8.5; phosphate and pyrophosphate buffers were used; below pH 5.5 the enzyme is unstable. Each tube contained 0.6 ml. of buffer, 0.15 ml. of 0.05 M maltose and 0.75 ml. of enzyme (160 mg. dissolved in 6 ml. of 1.92 mM riboflavin). Fig. 2 shows that the optimum synthesis of riboflavinyl glucoside from maltose and riboflavin is obtained at pH 6.7.

Glucosidase activity of the enzyme preparation

Breakdown of riboflavinyl glucoside. This was studied in the presence and in the absence of glucose, in order to differentiate between reaction mechanisms (2) and (3), as explained in the Discussion.

Fig. 1. Inhibition of the synthesis of riboflavinyl glucoside by α -D-glucose 1-phosphate. Curve 1, glucose 1-phosphate absent; curve 2, 1.50 mM glucose 1-phosphate present. Ordinate: reciprocal of rate of formation of riboflavinyl glucoside [$1/(\mu\text{g.}/\text{mg. enzyme/hr.})$]; abscissa: reciprocal of molar concentration of maltose.

Fig. 2. The effect of pH on the rate of synthesis of riboflavinyl glucoside.

Enzyme (50 mg.) was dissolved in 2.5 ml. of 0.1 M phosphate buffer, pH 7.4, and incubated in a Warburg manometer with 0.2 ml. of 0.1% notatin. A small uptake of O_2 ($15 \mu\text{l.}$) occurred during the first 5 min. after mixing the manometer contents, but no further uptake was observed during the next 30 min. Enzymically prepared riboflavinyl glucoside (1.5 ml. of 2.46 mM solution, dissolved in 0.1 M phosphate buffer, pH 7.4) was incubated in a manometer with 0.2 ml. of notatin at 38° ; no gas exchange occurred.

Enzyme solution (0.65 ml.) and 0.25 ml. of riboflavinyl glucoside solution were removed from the manometers and added to a tube containing 0.1 ml. of M glucose solution; similar amounts of enzyme and of riboflavinyl glucoside were added to a tube containing 0.1 ml. of water. The tubes were incubated at 35° and duplicate samples taken from each at 0, 0.5, 1 and 1.5 hr. Riboflavin was separated chromatographically from riboflavinyl glucoside and estimated fluorimetrically; no riboflavin was present in the samples taken at 0 hr. Riboflavin was formed both in the presence and absence of 0.1 M glucose, and the rate of formation of riboflavin was equal in the two experiments. It was confirmed by manometric measurement that the notatin had not completely oxidized the added glucose after 2 hr. at 35° .

The Michaelis constant for riboflavinyl glucoside in its breakdown to riboflavin was determined. Enzyme (80 mg.) was dissolved in 1.5 ml. of M phosphate buffer, pH 7.4, and 0.25 ml. added to tubes containing 0.75 ml. of riboflavinyl glucoside solution of different strengths; duplicate samples were taken at 0, 0.5, 1 and 1.5 hr. The Michaelis constant for riboflavinyl glucoside at 35° and pH 7.4 was found to be 0.18 mM.

Action on other glycosides. 0.025 M Solutions of maltose, cellobiose, lactose and α -methyl glucoside were incubated manometrically (total volume = 2.2 ml.) in 0.1 M phosphate buffer, pH 7.4, at 38° in the presence of enzyme (13 mg./ml.) and notatin (0.1 mg./ml.). With maltose, a steady uptake of oxygen occurred ($10.4 \mu\text{l.}/\text{hr.}$), but with the other sugars no uptake occurred after a small initial uptake of 8–12 $\mu\text{l.}$ in each case; the latter figure is approximately equivalent to the small reaction observed in the previous experiment where notatin was used to free the enzyme from glucose or glucose-forming substances. Control experiments showed that the notatin did not exhibit any maltase activity (cf. Whistler, Hough & Hylin, 1953).

Riboflavinyl β -D-glucoside, maltoflavin and enzymically prepared riboflavinyl glucoside were each added in sufficient amount to saturate 0.3 ml. of enzyme solution (4 mg. enzyme in 0.1 M phosphate buffer, pH 7.4) and the three solutions incubated at 35° (the strength of each flavin solution was approximately 6 mM). Samples (50 $\mu\text{l.}$ each) were taken at 0, 1.5, 3 and 4.5 hr., and the chromatograms developed with butanol:acetic acid:water (4:1:5, v/v). No formation of riboflavin was observed from riboflavinyl β -D-glucoside, but with maltoflavin ($R_F = 0.18$) as substrate a second fluorescent spot was detected ($R_F = 0.26$). This spot

was assumed to be glucoflavin and its intensity increased during the incubation. No sample of glucoflavin was available, but the isomeric galactoflavin has $R_F = 0.26$ in this solvent. No accurate comparison of the rates of breakdown of maltoflavin and of enzymically prepared riboflavinyl glucoside could be made, because of the small amount of maltoflavin available, but it was apparent that maltoflavin was being broken down much more slowly than enzymically prepared riboflavinyl glucoside; glucoflavin was formed from maltoflavin at less than 5% of the rate of formation of riboflavin, as judged by comparison of the intensities of fluorescence of their spots on the chromatograms.

Synthesis of glucosides from various isoalloxazine derivatives

Enzyme (420 mg.) was dissolved in 1.4 ml. of M phosphate buffer, pH 7.4, 7 ml. of 0.05M maltose and 5.6 ml. of water; 1.5 ml. of this solution was saturated with each of the flavins listed in the first column of Table 1. The experiment was performed at one concentration only with each flavin, because of shortage of these substrates, except for riboflavin where the Michaelis constants have been determined for reaction (1). Except with 9-oxyethyl-isoalloxazine, a second spot appeared on the chromatograms, and the rate of synthesis of this second spot is given in the third column of Table 1. The last column of Table 1 relates the rate of flavin glucoside synthesis to the concentration of the parent flavin.

The proof that the synthesis of a flavin glucoside is being measured in this experiment rests upon the following facts. If D-araboflavin, for instance, is incubated under these conditions without added maltose, the formation of only a minute amount of a second flavin can be detected on paper chromatograms, but the amount of this second flavin is greatly increased if maltose is added to the reaction mixture. D-Araboflavin is stable in the presence of

maltose if the enzyme has been previously inactivated by being boiled for 5 min., and no second spot can then be detected on chromatograms. The relative positions of D-riboflavin and riboflavinyl glucoside (for R_F values, see Whitby, 1952) are similar to the relative positions of D-araboflavin and its derived flavin when chromatographed in the same solvents (e.g. in butanol:acetic acid:water (4:1:5, v/v) and in water, saturated with isoamyl alcohol, D-araboflavin has R_F values 0.30 and 0.52, and its derived flavin has 0.20 and 0.60, respectively). D-Araboflavinyl glucoside has been made on a small scale, hydrolysed and the liberation of glucose demonstrated chromatographically (see below). Only in the case of riboflavinyl glucoside have the position and configuration of the glucosidic link been determined (Whitby, 1952); by analogy, it may be inferred that glucose is transferred from maltose to the terminal position of the polyhydroxylic side chains of the flavins studied in Table 1, to give an α -glucoside.

D-Araboflavinyl glucoside. Enzyme solution (2 ml.; 60 mg. of enzyme and 0.05M maltose in 0.2M phosphate buffer, pH 7.4) was saturated with D-araboflavin and incubated at 35° for 5 hr.; undissolved D-araboflavin was removed by centrifuging and the solution applied to a sheet of Whatman no. 3 paper (a blank sheet of paper was similarly treated in the subsequent steps). The enzyme was denatured, the chromatogram developed with water, saturated with isoamyl alcohol, and the faster-moving area ($R_F = 0.6$) was cut out, eluted with water at 70° and the solution evaporated to dryness. The residue was chromatographed a second time to ensure that all glucose and maltose had been removed (they ran near the solvent front) and, after elution with 10 ml. of water, the flavin solution was refluxed with an equal volume of NH_3 (sp.gr. 0.880) at 100° for 1 hr. to destroy the alkali-labile ring of the isoalloxazine nucleus (Kuhn & Rudy, 1934); treatment with NH_3 eliminated the intense yellow colour of flavin, which would otherwise have interfered with the chromatographic detection of glucose. Ammonia was removed *in vacuo* and the solid residue heated with 2 ml. of 2N-HCl at 100° for 2 hr.; the solution was cooled, passed through a column of Amberlite 1R-4B (acetate form), frozen at -20° and dried over P_2O_5 and

Table 1. *Synthesis of glucosides of various isoalloxazine derivatives*

In these reactions maltose was the glucose-donating substrate. For conditions, see the text.

Glucose-accepting substrate	Concentration (M $\times 10^4$) A	Glucoside synthesized (mole $\times 10^9$ /hr.)/mg. enzyme B	Expressed as percentage of the action with riboflavin B/A
D-Riboflavin	5.3	1.08	100
L-Araboflavin	6.8	1.30	94
D-Araboflavin	5.5	1.10	98
L-Lyxoflavin	0.55	0.11	98
D-Galactoflavin	7.0	0.02	1-2
isoRiboflavin	2.8	0.37	65
9-L-Arabityl-isoalloxazine	12.0	2.25	92
9-Dihydroxypropyl-isoalloxazine	2.5	0.16	31
9-Oxyethyl-isoalloxazine	2.1	0	0

NaOH. The residue was chromatographed in butanol:acetic acid:water (4:1:5, v/v) and the chromatogram sprayed with aniline hydrogen phthalate (Partridge, 1949); glucose was detected, but nothing was found in the blank, which had been treated similarly throughout in case Whatman no. 3 filter paper had yielded some glucogenic material under these conditions.

Enzymic synthesis of riboflavinyl glucoside from riboflavin and various glucose donors

The following sugars (0.03M) were incubated at pH 7.4 (0.1M phosphate buffer) with enzyme (13.3 mg./ml.) and riboflavin (1.72 mM) and found to be unable to replace maltose as glucose donor in reaction (1): α -D-glucose 1-phosphate, α -methyl glucoside, cellobiose, gentiobiose, isomaltose, lactose, melezitose, melibiose, methyl maltoside, panose, raffinose, sucrose and α -trehalose. The competitive inhibition of reaction (1) by α -glucose 1-phosphate has already been described. Under identical conditions it was found that maltulose (4-*o*- α -glucopyranosyl-D-fructose) and turanose (3-*o*- α -glucopyranosyl-D-fructose) were able to replace maltose in reaction (1), the relative rates of synthesis of riboflavinyl glucoside from maltose, maltulose and turanose being 10:9:6. The proof that riboflavinyl glucoside has been synthesized from riboflavin and maltulose or turanose rests on evidence similar to that advanced in the case of the flavin glucosides listed in Table 1. Riboflavinyl glucoside was prepared enzymically from riboflavin and turanose, degraded in the manner described for D-araboflavinyl glucoside, and the liberation of glucose detected chromatographically.

Solubility of riboflavinyl glucoside

Riboflavinyl glucoside was shaken with 0.1M phosphate buffer, pH 7.4, under toluene for 2 days at 20 and 37°. The solutions were centrifuged to remove undissolved riboflavinyl glucoside, and the strength of each solution was determined. Riboflavinyl glucoside was shown to be stable under these conditions, and neither riboflavin, lumiflavin nor lumichrome could be detected chromatographically. The solubilities at 20 and 37° were 2.2 and 3.5 mg./ml., respectively, which should be compared with the corresponding solubilities of riboflavin of 0.1 and 0.2 mg./ml., respectively, under these conditions.

DISCUSSION

The glucosidic link in enzymically synthesized riboflavinyl glucoside was not definitely shown to be an α -linkage by Whitby (1952), but the fact that the glucosidase investigated here was unable to hydrolyse synthetic riboflavinyl glucoside, which is believed to be a β -glucoside (Plant, 1952), coupled

with its ability to hydrolyse the enzymically synthesized compound under identical conditions, is further evidence for the latter being riboflavinyl α -D-glucoside.

The breakdown of riboflavinyl α -D-glucoside might proceed by either or both of the following routes:

Reaction (2a) would be catalysed by a transglucosidase and would require the presence of glucose to initiate it, whereas reaction (3) would be catalysed by a glucosidase. The enzyme preparation was shown to catalyse reaction (2b) so, to differentiate between reactions (2) and (3), it was necessary to remove glucose and glucose-forming substances from one set of solutions. This was effected by previous incubation with notatin, and the presence of notatin during the experiment would also have prevented reaction (2a) from becoming autocatalytic. The rate of breakdown of riboflavinyl α -D-glucoside was not reduced in the absence of glucose; from this it could be deduced that reaction (2a) was not obligatory for the breakdown of riboflavinyl α -D-glucoside, that reaction (3) took place, and that in all probability two different enzymes were not present (one to catalyse reaction (2a) and the other reaction (3)) since the rate of breakdown would then have been expected to be greater in the presence of glucose than in its absence. The experiment does not distinguish between the following possibilities: that two different enzymes catalyse the synthesis and the breakdown of riboflavinyl α -D-glucoside (a transglucosidase and a glucosidase respectively); that there is a single enzyme which has both transglucosidase and glucosidase activities. The latter possibility is favoured because of similarities in specificity between the transglucosidase and glucosidase activities of the enzyme preparation. Bealing & Bacon (1953) believe that the β -fructofuranosidase of *Penicillium spinulosum* has both transferase and hydrolase activities.

The transglucosidase will not synthesize a glucoside of 9-oxyethyl-isoalloxazine, which is a monohydric alcohol, nor will the glucosidase hydrolyse α -methyl glucoside, in which the aglycone is a monohydric alcohol. Secondly, Table 1 shows that the rate of synthesis of galactoflavinyl glucoside is slow relative to the rate of synthesis of riboflavinyl α -D-glucoside, and it was found that the glucosidase hydrolysed maltoflavin slowly when compared with riboflavinyl α -D-glucoside; these two experiments, although not entirely comparable,

since galactoflaviny l glucoside is thought to be the 6'-glucoside, whereas maltoflavin is the 4'-glucoside, do suggest that the pentityl side chain of riboflavin is more suited structurally to both glucosidase and transglucosidase activity than the hexityl side chains of galactoflavin and glucoflavin.

Table 1 shows that the transglucosidase is able to synthesize glucosides of several *isoalloxazine* derivatives, but the relative affinity of the enzyme for the different compounds cannot be deduced from the table because the comparison was made in terms of concentrations and not Michaelis constants. The low reactivity of galactoflavin is noteworthy because yeast flavokinase does not phosphorylate galactoflavin (Kearney, 1952) and because galactoflavin is an antivitamin (Emerson, Wurz & Johnson, 1945).

Maltose can be replaced by maltulose or turanose in reaction (1), but the relative affinity of the enzyme for these three disaccharides has not been assessed in terms of their Michaelis constants. The specificity requirements of the enzyme for the carbohydrate donor can be partly defined by considering the points of structure common to maltose, maltulose and turanose, and contrasting them with the many compounds which have been found to be unable to replace maltose in reaction (1). Only reducing disaccharides having an α -glucosidic link have been found to be active, and the inability of *isomaltose* and panose to replace maltose shows that the point of linkage between the monosaccharides is important. D-Glucose and D-fructose can function as aglycone, and possibly other compounds may do so too; only α -glucopyranosides have been found active (melibiose, an α -galactoside, was inactive, but it has the 1:6-linkage, like *isomaltose*), so the enzyme may be a transglycosidase or, more specifically, a transglucosidase.

Yeast invertase, acting on sucrose in the presence of methanol, was shown to produce methyl β -fructofuranoside (Bacon, 1952), and the formation of possibly analogous compounds was observed from three other monohydric alcohols. Bealing (1953) incubated mould extracts with sucrose or raffinose and various monohydric and dihydric alcohols, and observed the appearance of new fructose-containing spots on chromatograms; these were thought to be the corresponding alkyl β -fructofuranosides. Flavin glucosides are formed by a similar reaction from maltose and certain polyhydric *isoalloxazines*, but the enzyme differs in its specificity requirements from the enzymes studied by Bacon (1952) and by Bealing (1953) since it does not transfer glucose to the monohydric alcohol, 9-oxyethyl-*isoalloxazine*. It is possible that the proximity of the heterocyclic structure to the alcoholic group inhibited the enzyme completely, but this would not explain the rate of synthesis of

galactoflaviny l glucoside, which is very slow compared to the rate of synthesis of other flavin glucosides (Table 1). The enzyme which catalyses the synthesis of flavin glucosides must require other conditions to be fulfilled by the acceptor molecule besides the possession of a primary alcoholic group. Another difference, possibly, between this transglucosidase and the transfructosidases described by Bacon (1952) and Bealing (1953) is that three reducing disaccharides have been found to act as glucose donors with the former enzyme and three non-reducing disaccharides have been found to be inactive, whereas the non-reducing sugars sucrose or raffinose are required for the transfructosidations (no reducing disaccharide containing β -fructofuranose has been tested).

If the enzyme catalysing reaction (1) had been found to have an absolute specificity for D-riboflavin, it would have been possible to infer that riboflaviny l α -D-glucoside had a physiological function, but Table 1 shows that glucosides of antivitamin₈, such as D-araboflavin (Euler & Karrer, 1946) and *isoriboflavin* (Emerson & Tishler, 1943), can also be synthesized enzymically. However, yeast flavokinase phosphorylates D-araboflavin and dichloroflavin in addition to D-riboflavin (Kearney, 1952), and *isoriboflavin* is more effective in catalysing the non-enzymic reduction of cytochrome *c* by reduced pyridine nucleotides than the flavins which are known to occur naturally (Singer & Kearney, 1950*b*), so the enzymic synthesis of glucosides from antagonists of vitamin B₂ does not necessarily mean that riboflaviny l α -D-glucoside has no physiological significance. It is much more soluble in water than riboflavin and this property may be of importance in the transport of, perhaps, the least water-soluble of all the water-soluble vitamins. This suggestion would be more acceptable if riboflaviny l α -D-glucoside had been shown to occur naturally; it has not been observed in tissue extracts, but it may have been hydrolysed enzymically to riboflavin in the early stages of the isolation procedures adopted. Bessey, Lowry & Love (1949) estimated the amount of different flavins in various tissues and found that flavin mononucleotide and flavin-adenine dinucleotide comprised the major part of the total flavin. If riboflaviny l α -D-glucoside forms part of the unphosphorylated flavin in tissues, it can only be present in small amount, which may explain why it has not been detected.

SUMMARY

1. The specificity of the enzyme which synthesizes riboflaviny l glucoside has been investigated in an attempt to assess the physiological significance of this derivative of riboflavin. It is possible that the

enzyme has both transglucosidase and glucosidase activities.

2. The transglucosidase synthesizes glucosides of several isoalloxazine derivatives, many of which are antivitamin. Certain specificity requirements have been defined for the glucose donor and for the flavin acceptor.

3. The quantitative characteristics of the formation of riboflavinyl glucoside from riboflavin and maltose have been investigated, and the inhibition of this reaction by α -D-glucose 1-phosphate has been shown to be competitive.

4. The solubility of riboflavinyl glucoside in water is much greater than the solubility of riboflavin, and it is suggested that this may be of importance in the transport of riboflavin.

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Comparative Detoxication

3. HIPPURIC ACID FORMATION IN ADULT LOCUSTS

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It has been shown that the detoxication of phenols in the locust is accomplished by a conjugation mechanism different from that used by vertebrates, glucosides rather than glucuronides being formed (Myers & Smith, 1954). It was of interest, therefore, to consider the fate of aromatic acids in this insect, since even among vertebrates much variation is found in the method of conjugation of these acids, which may be condensed with glucuronic acid, glycine, ornithine, or glutamine in different species (Williams, 1947). Previous work has given very little indication of the existence of a conjugation mechanism in insects for the detoxication of aromatic acids though the reported presence of hippuric acid in some insects suggested that this might be possible. This identification of hippuric acid, in the faeces of various caterpillars (Davy, 1854; van der Hoeven, 1850), and in the fat body of *Dytiscus* (Sirodt, 1858) rests on microscopical observation of crystal form and is uncertain.

The presence of some conjugated benzoic acid

such as hippuric acid in the excreta of locusts, which feed on grass, might be expected since this compound is a typical urinary constituent of vertebrate herbivores. However, Brown (1937) was unable to find hippuric acid in the excreta of another Acridid, the grasshopper, *Melanoplus bivittatus*.

We have studied this detoxication mechanism by observing the metabolism of some aromatic acids whose glycine conjugates can readily be detected in the excreta, and it will be shown that the locust, like most animals, conjugates the aromatic acids with glycine to form hippuric acids.

EXPERIMENTAL

Reference compounds. *p*-Nitrohippuric acid, m.p. 135°, and dibenzoylornithine (ornithuric acid), m.p. 185°, were prepared by the Schotten-Baumann reaction using the appropriate amino acids and acyl chlorides. *o*-Hydroxyhippuric acid (salicyluric acid), m.p. 166°, was prepared from salicylic azide according to Bondi (1907). Hippuric acid, m.p. 186°, and *p*-aminohippuric acid, m.p. 199°, were com-