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HERBICIDE METABOLISM IN PLANTS—I.

PURIFICATION AND PROPERTIES OF UDP-GLUCOSE:ARYLAMINE N-GLUCOSYL-TRANSFERASE FROM SOYBEAN*

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Abstract—A "soluble" enzyme system from soybean, which catalyzes the biosynthesis of *N*-glucosylarylamines, has been purified 20-fold by differential centrifugation, ammonium sulfate fractionation, gel filtration, and cellulose ion exchange chromatography. The enzyme was specific for the nucleotide glucosyl donors uridinediphosphate glucose (UDPG) and thymidinediphosphate glucose (TDPG), but exhibited a broad specificity toward acceptor arylamines. A number of substituted anilines and aminobenzoic acids were studied as acceptor arylamines including 2,5-dichloro-3-aminobenzoic acid (amiben) and 3,4-dichloroaniline, a metabolite of 3,4-dichloropropionanilide (propanil) in higher plants. The partially purified enzyme was found to have an optimum pH of 7·5 with 3,4-dichloroaniline as the arylamine acceptor and was inhibited by sulfhydryl reagents. The Km constants for UDPG and 3,4-dichloroaniline were $1{\cdot}88 \times 10^{-3}$ M and $5{\cdot}63 \times 10^{-4}$ M respectively. The Ki constant for uridinediphosphate (UDP) was $4{\cdot}84 \times 10^{-4}$ M.

INTRODUCTION

RECENT studies with infiltrated plant tissue sections have demonstrated the extensive and enzymatic nature of *N*-(3-carboxy-2,5-dichlorophenyl)-glucosylamine formation from the corresponding arylamine.[1] This paper is a report of the partial purification and characterization of an enzyme from soybean which catalyzes the biosynthesis of *N*-glucosylarylamines and a discussion of the relationship of this enzyme to the metabolism of a variety of herbicides in plants.

RESULTS AND DISCUSSION

Distribution of UDP-glucose: arylamine N-*glucosyltransferase in Etiolated Soybean Seedlings*

Table 1 shows the results of an enzyme distribution study. The enzyme was present in all of the tissues examined, but was most active in the cotyledons and in acetone powders of the seed. These results are in agreement with the distribution pattern reported by Frear *et al.*[1] on the biosynthesis of *N*-(3-carboxy-2,5-dichlorophenyl)-glucosylamine in etiolated soybean tissue sections infiltrated with 3-amino-2,5-dichlorobenzoic acid.

Partial Purification of UDP-glucose: arylamine N-*glucosyltransferase from Soybean*

The results of a typical purification experiment are shown in Table 2 where a purification of over 19-fold was obtained with better than a 40 per cent recovery. No loss in enzyme activity was observed after centrifugation at up to 150,000 *g* for 90 min. Routinely, however, the

* Use of trade names is for the purpose of identification of equipment employed and does not constitute endorsement by the U.S. Department of Agriculture.

[1] D. S. FREAR, C. R. SWANSON and R. E. KADUNCE, *Weeds* **15** (2), 101–104 (1967).

crude enzyme was centrifuged at only 75,000 *g* for 30 min to remove the larger particulate matter. Ammonium sulfate was found to be inhibitory as shown by an increase in enzyme recovery after gel filtration. Similar results were observed in dialysis experiments.

TABLE 1. DISTRIBUTION OF UDP-GLUCOSE:ARYLAMINE *N*-GLUCOSYLTRANSFERASE IN ETIOLATED SOYBEAN SEEDLINGS

Tissue	*N*-(3,4-dichlorophenyl) glucosylamine formed (mμmoles/hr)	Protein (mg)	Specific activity (units/mg protein)
Roots	3·7	12·2	0·30
Hypocotyls	6·4	11·1	0·58
Cotyledons	33·5	9·2	2·55
Leaves	3·3	10·9	0·30
Seeds (acetone powder)	30·2	11·0	2·75

Six-day-old etiolated soybean seedling tissues were lyophilized, ground to pass a 30 mesh screen, extracted with an appropriate volume of 0·1 M potassium phosphate buffer pH 7·5 containing 1×10^{-2} M cysteine, and centrifuged at 75,000 *g* for 30 min to provide a crude cell-free enzyme preparation of 30–60 mg of protein per ml. The standard assay system was used with approximately 10 mg of protein per reaction mixture. Reaction and control tubes without UDPG were incubated for 3 hr at 25°. One unit = 1 mμmole *N*-(3,4-dichlorophenyl)-glucosylamine/hr.

An apparent separation of *O*-glucosyltransferase[2, 3] and *N*-glucosyltransferase activities found in cruder preparations was achieved by cellulose ion exchange chromatography. This is illustrated in Table 3 by a marked change in the specific activity ratios before and after ion exchange chromatography.

Stability

Storage of the dissolved 30–60 per cent ammonium sulfate fraction for 48 hr at 4° or lyophilization and storage for a week at −15° resulted in a 50 per cent loss in activity. Lyophilized G-50 fractions, on the other hand, were stable for several months at −15°. Avigad and Milner[4] have recently reported that UDP-glucose:D-fructose transglucosylase from sugar beet roots is also inactivated on prolonged incubation with high concentrations of $(NH_4)_2SO_4$ or phosphate. Freezing and thawing of the Sephadex G-50 fraction produced no loss of activity but heating for 10 min at 50° resulted in an 80 per cent loss of activity. Attempts to fractionate the enzyme with organic solvents (acetone and ethanol) also resulted in almost complete inactivation of crude cell-free preparations.

Optimum pH

The pH optimum of N-(3,4-dichlorophenyl)-glucosylamine formation by partially-purified soybean UDP-glucose:arylamine *N*-glucosyltransferase was found to be between 7·4 and 7·8. Tris buffer [2-amino-2-(hydroxymethyl)-1,3-propanediol] with a free primary amine group was found to be inhibitory and resulted in only 83 per cent of the activity of bicine [*N,N-bis*(2-hydroxyethyl)glycine] or phosphate buffers at pH 7·6.

[2] T. YAMAHA and C. E. CARDINI, *Arch. Biochem. Biophys.* **86**, 127 (1960).
[3] J. B. PRIDHAM and M. J. SALTMARSH, *Biochem. J.* **87**, 218 (1963).
[4] G. AVIGAD and Y. MILNER, *Methods in Enzymology*, Vol. VIII, p. 341, Academic Press N.Y. (1966).

TABLE 2. PURIFICATION OF SOYBEAN UDP-GLUCOSE:ARYLAMINE *N*-GLUCOSYLTRANSFERASE

Fraction	Volume (ml)	Protein (mg/ml)	Units*/ml	Total units	Specific activity (units/mg protein)	Recovery (%)	Purification (fold)
1. 1,000 *g* supernatant	130·0	47·3	133·3	17,329	2·82	100·0	—
2. 75,000 *g* supernatant	123·0	46·5	148·1	18,216	3·19	105·1	1·13
3. 30–60 per cent $(NH_4)_2SO_4$	25·6	88·6	534·9	13,698	6·04	79·0	2·14
4. Sephadex G-50	97·6	20·8	201·6	19,670	9·69	113·5	3·44
5. DEAE-cellulose	16·1	8·8	481·5	7,752	54·72	44·7	19·40

* 1 unit = 1 mμmole N-(3,4-dichlorophenyl)-glucosylamine/hr.

TABLE 3. SEPARATION OF *O*- AND *N*-GLUCOSYLTRANSFERASE ACTIVITIES ON DEAE CELLULOSE

Fraction	Specific activity (units*/mg protein)		Purification (fold)		Specific activity ratio
	HQ†	3,4-DCA‡	HQ	3,4-DCA	HQ/3,4-DCA
Sephadex G-50	36·7	6·6	—	—	5·56
DEAE-cellulose	49·5	50·2	1·41	7·6	0·99

* 1 unit = 1 mμmole of glucoside/hr.
† Hydroquinone as substrate.
‡ 3,4-dichloroaniline as substrate.

Inhibition Studies

The effect of a number of inhibitors upon enzyme activity are shown in Table 4. No inhibition was observed at the 1·0 mM level with NaF, NaCN, NaN_3 and Na_2HAsO_4. Several thiol inhibitors, on the other hand, were found to be very effective inhibitors. Except for a decreased inhibition by iodoacetamide and iodoacetate, these results agree with earlier inhibitor studies on the biosynthesis of *N*-(3-carboxy-2,5-dichlorophenyl)-glucosylamine in

TABLE 4. INHIBITION OF SOYBEAN UDP-GLUCOSE-ARYLAMINE *N*-GLUCOSYLTRANSFERASE

Inhibitor	Concentration (mM)	Inhibition %
p-Chloromercuribenzoate	0·5	100
	0·1	11
N-Ethylmaleimide	0·5	82
	0·1	4
o-Iodosobenzoate	0·5	82
	0·1	9
p-Benzoquinone	0·5	98
	0·1	10
Catechol	1·0	22
	0·5	10
Glutathione (oxidized)	1·0	14
Iodoacetamide	1·0	10
Iodoacetate	1·0	4
$NaAsO_2$	1·0	17
$HgCl_2$	0·5	100
	0·1	56
$CuSO_4$	0·5	98
	0·1	34
EDTA	1·0	2

The complete reaction mixture contained 100 μmoles phosphate buffer pH 7·5, 2·0 μmoles UDPG, 0·5 μmoles 3,4-dichloroaniline, inhibitor, 9–11 mg Sephadex G-50 enzyme, and distilled water to a final volume of 0·50 ml. The inhibitors were incubated for 15 min at 25° with enzyme, buffer and distilled water before the addition of the substrates UDPG and 3,4-dichloroaniline. The reaction was then incubated for 3 hr at 25°. Controls were run without UDPG.

infiltrated soybean tissue sections.[1] The increased inhibition by iodoacetamide and iodoacetic acid in tissue sections may be largely due to an indirect effect of these inhibitors on available uridinediphosphate (UDP) glucose pools in these tissues rather than a direct effect on the enzyme. Inhibition by *p*-benzoquinone and catechol were similar to the results of C. R. Slack[5] with UDP-glucose:D-fructose 2-glucosyltransferase from sugarcane except that the soybean UDP-glucose:arylamine *N*-glucosyltransferase did not appear to be as sensitive to catechol inhibition. This may be a reflection of a lower phenol oxidase activity in the partially purified soybean enzyme preparations used.

Substrate Specificity

Only UDPG and TDPG were found to be effective glucosyl donors for *N*-(3,4-dichlorophenyl)-glucosylamine formation with soybean UDP-glucose arylamine *N*-glucosyltransferase as shown in Table 5. Similar results were obtained with 3-amino-2,5-dichlorobenzoic

TABLE 5. NUCLEOTIDE SPECIFICITY OF SOYBEAN UDP GLUCOSE:ARYLAMINE *N*-GLUCOSYLTRANSFERASE

Glucosyl donor	Relative activity (%)
UDPG	100
TDPG	37
GDPG	<1
CDPG	0
ADPG	0

The complete reaction mixture contained 100 μmoles phosphate buffer pH 7·5, 2·0 μmoles of appropriate glucosyl donor, 0·5 μmoles of 3,4-dichloroaniline, 9·0 mg Sephadex G-50 enzyme, and distilled water to a final volume of 0·50 ml. Reaction mixtures were incubated for 3 hr at 25°. Controls were run in the absence of glucosyl donor.

acid as the arylamine acceptor. Uridinediphosphate galactose was found to form an *N*-(3,4-dichlorophenyl)-glycosylamine. Gas chromatographic analysis of the hydrolyzed carbohydrate moiety according to the procedure of Richey *et al.*[6] demonstrated the presence of only glucose. Apparently the partially purified soybean UDP-glucose:arylamine *N*-glucosyltransferase preparation used in these experiments also contained a UDP-glucose 4′-epimerase. No *N*-(3,4-dichlorophenyl)-glycosylamine was formed with UDP mannose as the glycosyl donor and 3,4-dichloroaniline as the arylamine acceptor.

Specificity toward arylamine acceptors was found to be rather broad as shown in Table 6. It appears that the relative activity of the various substrates may be related to the electron density about the amino nitrogen as shown by the decreased activity of *p*-chloroaniline compared with *m*-chloroaniline and of *m*-chloroaniline compared with *m*-nitroaniline. Decreased activity of *o*-chloroanilines may also involve the influence of a steric effect as evidenced by the reduced activity of *o*-chloroaniline compared with *p*-chloroaniline and of 2,6-dichloroaniline compared with 2,4-dichloroaniline.

[5] C. R. SLACK, *Phytochem.* **5**, 397 (1966).
[6] J. M. RICHEY, H. G. RICHEY and R. SCHRAER, *Anal. Biochem.* **9**, 272 (1964).

TABLE 6. SUBSTRATE SPECIFICITY OF SOYBEAN UDP-GLUCOSE:ARYLAMINE *N*-GLUCOSYLTRANSFERASE

Substrate	Relative activity (%)
3,4-Dichloroaniline	100
m-Chloroaniline	74
p-Chloroaniline	31
p-Bromoaniline	28
m-Nitroaniline	18
2,4-Dichloroaniline	18
2,5-Dichloroaniline	17
o-Chloroaniline	16
2,3-Dichloroaniline	16
2,4-Dibromoaniline	12
2,6-Dichloroaniline	7
3,5-Dichloroaniline	6
3-Amino-2,5-dichlorobenzoic acid	6
m-Aminobenzoic acid	6
5-Amino-2,3-dichlorobenzoic acid	4

Relative activities of *o*-nitroaniline, *p*-nitroaniline, 4-amino-2,5-dichlorobenzoic acid, and 6-amino-2,5-dichlorobenzoic acid were all less than 2%. The complete reaction mixture contained 100 μmoles phosphate buffer pH 7·5, 2·0 μmoles UDPG, 0·5 μmoles arylamine substrate, 8–11 mg Sephadex G-50 enzyme, and distilled water to a final volume of 0·50 ml. Reaction mixtures were incubated for 3 hr at 25°. Controls were run in the absence of UDPG.

Recent papers have shown that several arylamine herbicides are metabolized directly to *N*-glucosides by plants. These include 5-amino-4-chloro-2-phenyl-3(2H)-pyridazinone (pyrazon),[7,8] 3-amino-1,2,4-triazole (amitrole)[9,10] and 3-amino-2,5-dichlorobenzoic acid.[1,11–14] In addition to direct conjugation with intact arylamine herbicides, two herbicides, 3-nitro-2,5-dichlorobenzoic acid (dinoben)[15] and 3,4-dichloropropionanilide,[16] have recently been shown to be metabolized to arylamines which then are conjugated with glucose. A number of other herbicides have been reported to be metabolized to arylamines by plants, but their respective glucosides have not been isolated and identified. These herbicides include the carbamates, isopropyl-*N*-(3-chlorophenyl)carbamate (CIPC)[17,18] and 4-chloro-2-butynyl-*N*-(3-chlorophenyl) carbamate (Barban),[19] and the ureas *N'*-(4-chlorophenoxy)-

[7] S. K. RIES, G. R. STEPHENSON and M. J. ZABIK, *Weed Abstr.* 67 (1967).
[8] S. K. RIES, M. J. ZABIK, G. R. STEPHENSON and T. M. CHEN, *Weeds* (in preparation).
[9] B. J. ROGERS, *The Hormolog*, Department of Botany and Plant Physiology, Purdue University, Lafayette, Indiana (1957).
[10] J. F. FREDRICK and A. C. GENTILE, *Arch. Biochem. Biophys.* **92**, 356 (1961).
[11] S. R. COLBY, *Weed Abstr.* 68 (1967).
[12] S. R. COLBY, *Science* **150**, 619 (1965).
[13] C. R. SWANSON, R. H. HODGSON and D. S. FREAR, *Plant Physiol.* (Suppl.) **40**, XIV, Abstr. (1965).
[14] C. R. SWANSON, R. E. KADUNCE, R. H. HODGSON and D. S. FREAR, *Weeds* **14**, 319 (1966).
[15] S. R. COLBY, Division of Agricultural and Food Chemistry Abstr. 152nd Meeting of the American Chemical Society, New York 1966.
[16] G. G. STILL and R. E. KADUNCE, *Weed Abstr.* p. 64 (1967).
[17] P. C. KEARNEY, *J. Agri. Food Chem.* **13**, 561 (1965).
[18] R. H. HODGSON, *Weed Abstr.* p. 65 (1967).
[19] J. R. RIDEN and T. R. HOPKINS, *J. Agri. Food Chem.* **10**, 455 (1962).

phenyl-*N*,*N*-dimethylurea (chloroxuron)[20] and 3-(4-chlorophenyl)-1,1-dimethylurea (monuron.)[21,22] It therefore appears that the biosynthesis of *N*-glucosylarylamines may be a general pathway in the metabolism of many herbicides by plants.

Kinetic Studies

Double reciprocal plots of initial velocities at non-saturating concentrations of UDPG or 3,4-dichloroaniline yielded a family of straight lines which intersected at a point to the left of the ordinate and slightly below the abscissa as shown in Figure 1. According to the report of Morrison[23] such results show that the soybean UDP glucose:arylamine *N*-glucosyltransferase mechanism is sequential. Kinetic data were processed on an IBM 1620 computer

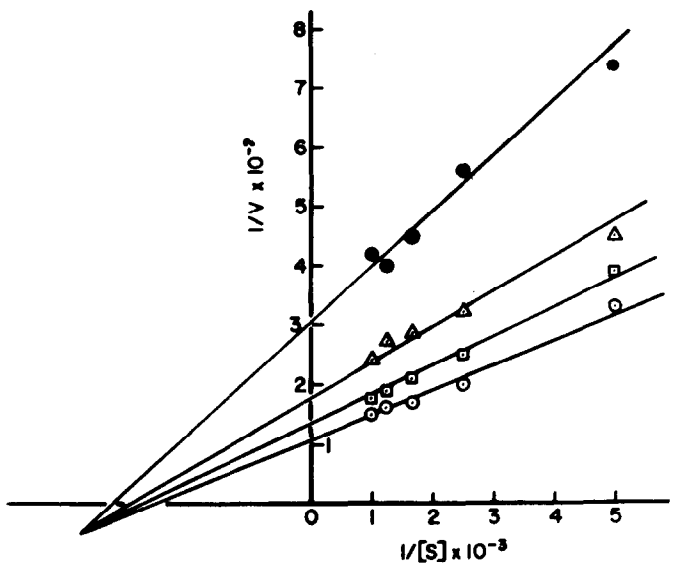

FIG. 1. DOUBLE RECIPROCAL PLOT OF 3,4-DICHLOROANILINE CONCENTRATION AGAINST RATE OF *N*-(3,4-DICHLOROPHENYL)-GLUCOSYLAMINE FORMATION.

The complete reaction mixture contained 100 μmoles phosphate buffer pH 7·5, 0·1–0·5 μmoles 3,4-dichloroaniline, 0·25–1·5 μmoles UDPG, 9·5 mg Sephadex G-50 enzyme and distilled water to a final volume of 0·50 ml. Reaction mixtures were incubated for 3 hr at 25°. Controls were run in the absence of UDPG. ●–●, 5×10^{-4} M UDPG; △–△, 10×10^{-4} M UDPG; □–□, 20×10^{-4} M UDPG; ⊙–⊙, 30×10^{-4} M UDPG.

using a sequential bireactant mechanism program of Cleland.[24,25] The Km constants for 3,4-dichloroaniline and UDPG were found to be $5{\cdot}63 \times 10^{-4}$ M and $1{\cdot}88 \times 10^{-3}$ M respectively. Uridinediphosphate was found to be a linear competitive inhibitor of the enzyme when UDPG was the variable substrate and 3,4-dichloroaniline was the fixed substrate held at non-saturating concentrations. The Ki value for UDP under these conditions was found to be

[20] H. C. GEISSBÜHLER, H. AEBI HASELBACH and L. EBNER, *Weed Res.* 3, 277 (1963).
[21] T. J. SHEETS and J. W. SMITH, Division of Agricultural and Food Chemistry Abstr. 152nd meeting of the American Chemical Society, New York, 1966.
[22] C. R. SWANSON and H. R. SWANSON, *Weed Sci.* (in press).
[23] J. F. MORRISON, *Australian J. Science* **27**, 317 (1965).
[24] W. W. CLELAND, *Nature* **198**, 463 (1963).
[25] W. W. CLELAND, In *Advances in Enzymology* Vol. 29, p. 1–32, Interscience, New York (1967).

$4{\cdot}84 \times 10^{-4}$ M when the data were processed on the computer using the linear competitive program of Cleland.[24, 25]

MATERIALS AND METHODS

Preparation of Acetone Powders

Soybeans (*Glycine max Merril*, var. Hawkeye) were ground in a Wiley cutting mill to pass through a 30 mesh screen. The resulting soybean meal was then extracted two times with 10 volumes of acetone at $-15°$, filtered, air dried at 4° and stored under vacuum over conc. H_2SO_4 at 4°.

Enzyme Extraction and Partial Purification

20 g of soybean acetone powder were extracted with 8 vol of 0·1 M K phosphate buffer at pH 7·5 containing 0·01 M cysteine. The resulting slurry was strained through cheesecloth and centrifuged at 1000 ***g*** for 15 min. The crude cell-free supernatant was centrifuged at 75,000 ***g*** for 30 min to remove the larger particulate matter. After centrifugation at 75,000***g*** the clarified supernatant was fractionated between 30 and 60 per cent saturation by the addition of crystalline enzyme grade $(NH_4)_2SO_4$. The protein precipitate formed at 60 per cent $(NH_4)_2SO_4$ saturation was collected by centrifugation at 10,000 ***g*** for 10 min and dissolved in a minimal amount of 0·05 M potassium phosphate buffer pH 7·5.

The dissolved 30–60 per cent $(NH_4)_2SO_4$ fraction was layered on top of a $2{\cdot}5 \times 38$ cm Sephadex G-50 column equilibrated with 0·05 M K phosphate buffer pH 7·5 containing 0·2 M NaCl. The enzyme was then eluted with approximately two void volumes of the equilibrating buffer at a flow rate of 1 ml per min. After elution from the Sephadex G-50 column, the enzyme was lyophilized in 20 ml ampules, sealed under vacuum and stored at $-15°$. Further purification of the enzyme was achieved by ion exchange chromatography on a $2{\cdot}5 \times 35$ cm DEAE cellulose column equilibrated with 0·05 M potassium phosphate buffer at pH 8·0. Approximately 500 mg of protein from the lyophilized Sephadex G-50 eluate was dissolved in 5·0 ml of distilled water, dialyzed against 4 l. of 0·05 M K phosphate buffer at pH 8·0 for 2 hr, centrifuged for 10 min at 10,000 ***g***, and placed on the DEAE column. Elution was carried out with a linear pH and ionic strength gradient using 0·05 M K phosphate buffer pH 8·0 and 0·05 M K phosphate buffer pH 6·86 with 0·6 M NaCl at a flow rate of 1 ml/min. The bulk of the enzyme activity was found in the second protein peak (peak II) eluted off the column between 125–225 ml. The 260 nm/280 nm absorption ratio of the eluate was 0·63–0·65 . The peak fractions were pooled, precipitated at 60 per cent saturation with $(NH_4)_2SO_4$, centrifuged at 10,000 ***g*** for 10 min, dissolved in a minimal volume of 0·1 M K phosphate buffer pH 7·5 and dialyzed against 1 l. of 0·1 M K phosphate buffer pH 7·5 for 2 hr. After dialysis the enzyme was assayed or lyophilized and stored in sealed ampules under vacuum at $-15°$. All steps in the extraction and purification of the enzyme were carried out at 0–4°.

Enzyme Assays

The UDP-glucose:arylamine *N*-glucosyltransferase assay was based on the rate of *N*-glucoside formation. The standard reaction mixture contained 100 μmoles of K phosphate buffer pH 7·5, 2·0 μmoles of UDPG, 0·5 μmoles of arylamine substrate, enzyme and distilled water to a final volume of 0·50 ml. The reaction was initiated by the addition of substrate and incubated at 25°. Controls were run without UDPG in the reaction mixture or with enzyme, buffer and distilled water heated in a boiling water bath for 10 min and cooled to room

temperature prior to the addition of UDPG and arylamine substrates. The reaction was stopped by the addition of 1·0 ml of methanol. All enzyme purification, pH optimum, nucleotide sugar specificity, inhibitor and kinetic studies were carried out with 3,4-dichloroaniline as the arylamine substrate because of its much greater reactivity, ease of hydrolysis and the availability of known synthetic *N*-(3,4-dichlorophenyl)-glucosylamine.[26] Under the conditions of the assay, enzyme activity was linear with time and proportional to protein concentration.

Assays for *O*-glucosyltransferase activity were carried out in the same manner as with UDP-glucose:arylamine *N*-glucosyltransferase except that hydroquinone was used as the glucosyl acceptor and the rate *O*-glucoside (arbutin) formation was determined.

The enzyme unit (U) was defined as that amount of enzyme required for the biosynthesis of 1 mμmole of *N*- or *O*-glucoside per hr under the conditions of the assay.

Protein was determined either by the method of Lowry *et al.*[27] using crystalline bovine serum albumin as the standard, or by the u.v. absorption method of Warburg and Christian.[28] In column techniques, the absorbance at 254 nm was measured to estimate the distribution of protein in eluate fractions.

Chromatographic Separation of the Glucoside Formed

The methanolic reaction mixtures were centrifuged at 1,000 *g* for 5 min and suitable aliquots of the clarified supernatants (500 or 1000 μl) streaked on 8 × 8 in. 375μ silica gel HF thin layer chromatographic plates. At the same time, 5–10 μg of the appropriate known reference compounds were also spotted on each thin layer plate.

The *N*-glucosides were separated from any remaining arylamine substrate by thin layer chromatography during either a 10 or 15 cm run with a butanol-ethanol-ammonia (2:1:1) solvent system. Most of the arylamines and their respective *N*-glucosides were easily located on the developed chromatograms as dark bands under ultraviolet light. The arylamines and their respective *N*-glucosides could also be located on the developed chromatograms by spraying the known reference spot areas on each plate with a fresh methanol-HCl (1:1) solution followed by a fresh solution of 1 per cent $NaNO_2$ in 1N HCl and then with a 1 per cent solution of *N*-naphthylethylenediamine dihydrochloride in 2N HCl.

Arbutin was separated from any remaining hydroquinone by thin layer chromatography during a 15 cm run with a butanol-ethanol-water (4:2:1) solvent system. The arbutin and hydroquinone areas were located on the developed chromatograms by spraying the known reference spot areas on each chromatogram with Millons Reagent.[29]

Quantitative Determination of Glucosides Formed

After the glucosides were separated and located on the thin layer chromatograms, they were scraped off the chromatographic plate into 1 × 15 cm chromatography columns containing glass wool plugs and 2 ml of acid washed, ignited sand. The glucosides were then eluted off the silica gel HF, hydrolyzed, and the aglucone determined colorimetrically.

The arylamine glucosides were eluted off the silica gel HF with 3 to 6 ml of methanol or methanol-HCl (10:1) into either 5 or 10 ml of acid solution (8 volumes of 1N HCl and 1

[26] R. E. KADUNCE, *J. Chromatog.* **30**, 204 (1967).
[27] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[28] O. WARBURG and W. CHRISTIAN, *Biochem. Z.* **310**, 384 (1942).
[29] E. MILLON, *Compt. rend.* **28**, 40 (1949).

volume of glacial acetic acid) and hydrolyzed for 1 hr at 80°. After cooling to room temperature, 0·5 ml of fresh 1 per cent $NaNO_2$ nitrite was added with mixing. 10 min later, 1·0 ml of 10 per cent ammonium sulfamate was added to remove excess nitrite. The resultant diazonium salt of the arylamine was allowed to stand 10 min and then coupled with 0·5 ml of 1 per cent *N*-naphthylethylenediamine dihydrochloride. The solution was adjusted to 10 or 25 ml with distilled water, filtered through a 1·2 μ RAWP millipore membrane filter and read at the appropriate wavelength of maximum absorption. With this modified Riden and Hopkins[19] procedure, quantitative results were obtained by comparison with standard curves of the arylamines used. Recoveries of known *N*-glucosides were quantitative under the conditions used.

Arbutin was eluted off the silica gel HF with three 2 ml portions of distilled water. The colorimetric determination of arbutin was carried out essentially according to the procedure of Yamaha and Cardini.[2] The eluted arbutin was reacted with 0·5 ml of 2N Folin-Ciocalteau phenol reagent and 2·0 ml of 20 per cent Na_2CO_3 in a boiling water bath for 1 min. The reaction mixture was then cooled, adjusted to 10 ml volume with distilled H_2O, filtered through a millipore RAWP 1·2 μ filter and read at 650 nm. A standard curve obeyed the Beer-Lambert Law from 0–50 μg of arbutin and recoveries off the chromatographic plates were quantitative.

Reagents

All arylamine substrates used were purified by recrystallization, sublimation or preparative thin layer chromatography and found to be homogeneous on thin layer chromatography or gas chromatography.[30, 31] The *N*-glucosides of amiben, 3,4-dichloroaniline and *m*-chloroaniline were prepared according to Kadunce[26] and purified by recrystallization and preparative thin layer chromatography. Nucleotides and nucleotide sugars were either Sigma Chemical Company or Calbiochem Corporation products. Amiben was a gift from Amchem Products, Inc. Bicine [*N*,*N*-bis (2-hydroxyethyl) glycine] was purchased from Calbiochem Corp. Whatmann diethylaminoethyl cellulose (DE32 or 52) was obtained from Reeve Angel. Sephadex G-50 was purchased from Pharmacia Fine Chemicals. Synthetic arbutin was a gift from S. B. Penick and Company. All other reagents were commercial products of analytical reagent grade.

Acknowledgements—I thank Miss Sharon Smylie for her technical assistance throughout the course of this work.

[30] K. J. BOMBAUGH, *Anal. Chem.* **37**, 72 (1965).
[31] H. G. HENKEL, *J. Gas Chromatog.* **3**, 320 (1965).