Phytochemistry, 1971. Vol. 10, pp. 2279 to 2286. Pergamon Press. Printed in England.

PURIFICATION AND PROPERTIES OF A UDP GLUCOSE: THIOHYDROXIMATE GLUCOSYLTRANSFERASE FROM HIGHER PLANTS*

MITSUYOSHI MATSUO† and E. W. UNDERHILL‡

National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan, Canada

(Received 16 October 1970, in revised form 22 December 1970)

Abstract-An enzyme which catalyses the formation of desulfobenzylglucosinolate by glucosyl transfer from UDP glucose to phenylacetothiohydroximate has been isolated from leaves of *Tropaeolum majus* L. and purified 20-fold. The enzyme possessed a high degree of specificity for the sugar acceptor molecule (thiohydroximates), the donor nucleotide (UDP) and its sugar (glucose). The activity of the enzyme was increased by sulfhydryl, chelating and reducing compounds: the activity disappeared in the absence of β-mercaptoethanol and was strongly inhibited by mercuric chloride and p-chloromercuribenzoate. Cell-free extracts of other glucosinolate-containing plants, including *Sinapis alba* L., *Nasturtium officinale* R. Br. and *Armoracia lapathifolia* Gilib. contained a similar glucosyl-transferase activity. It is concluded that the glycosylation of phenylacetothiohydroximate is an integral step in the biosynthesis of benzylglucosinolate.

INTRODUCTION

IN A PREVIOUS publication from this laboratory[1] it was demonstrated that the tracer from labeled phenylacetothiohydroximate§ (PATH)‖ and from desulfobenzylglycosinolate was incorporated into the mustard oil glucoside, benzylglucosinolate, with high efficiency. Based on these and earlier findings, a biosynthetic pathway for the formation of this glucosinolate was proposed (Fig. 1). PATH could only be considered as a tentative intermediate in this sequence since it had not been established that it was a naturally occurring compound in *Tropaeolum majus,* the plant employed in these studies.

Recently, we have established that PATH is in fact a compound present in *T. majus,* and that it is derived in this plant from phenylacetaldoxime,[5] a known[6,7] intermediate of benzylglucosinolate. An enzyme was also detected in extracts of *T. majus* which catalysed the formation of desulfobenzylglucosinolate by transglycosylation of PATH from UDPG.[5]

* This work is part XIII of a series on the biosynthesis of mustard oil glucosides. For paper XII see Ref. 5.

† Postdoctorate Fellow, National Research Council of Canada, 1968-1970. Present address: National Institute of Radiological Sciences, Anagawa, Chiba-shi, Japan.

‡ Person to whom requests for reprints should be addressed.

§ This nomenclature is regarded as more satisfactory than thiohydroximate.[2-4]

‖ Abbreviations used in this paper are: PATH (sodium phenylacetothiohydroximate), MCE (β-mercaptoethanol), ADPG (adenosine diphosphate glucose), CDPG (cytidine diphosphate glucose), GDPG (guanosine diphosphate glucose), TDPG (thymidine diphosphate glucose).

[1] E. W. UNDERHILL and L. R. WETTER, *Plant Physiol. 44,584 (1969).*
[2] O. EXNER and B. KAKAC, *Coll. Czechoslov. Chem.* Commun. **28,** 1956 (1963).
[3] K. NAGATA and S. MIZUKAMI, *Chem. Pharm. Bull. (Tokyo) 14 1255 (1966).*
[4] K. A. JENSON, O. BUCHARDT and C. CHRISTOPHERSEN, *Acta Chem. Scand.* 21, 1936 (1967).
[5] M. MATSUO and E. W. UNDERHILL, *Biochem. Biophys. Res. Commun. 36, 18 (1969).*
[6] B. A. TAPPER and G. W. BUTLER, Arch. *Biochem. Biophys.* 120,719 (1967).
[7] E. W. UNDERHILL, *European J. Biochem. 2, 61 (1967):*

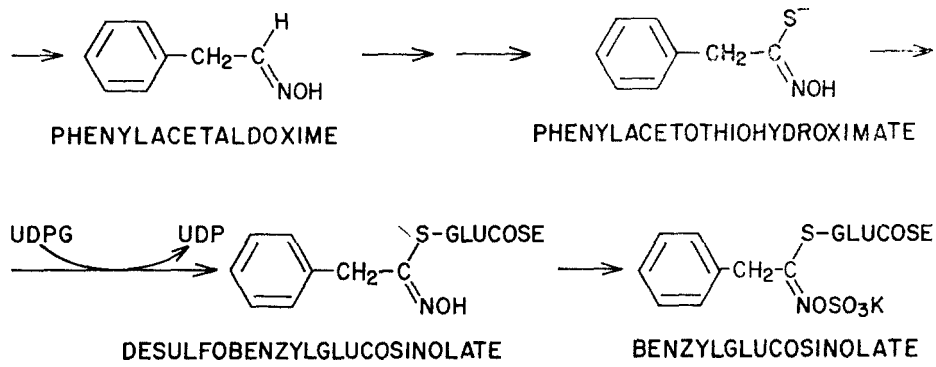

FIG. 1. PARTIAL PATHWAY FOR THE BIOSYNTHESIS OF BENZYLGLUCOSINOLATE.

To determine if the glucosylating enzyme is specific for the thiohydroximate precursor of benzylglucosinolate or if it merely catalyses a nonspecific detoxification reaction, we partially purified this protein fraction from the leaves of ***T. majus*** and studied some of its properties. In addition, we have isolated a similar enzyme from the leaves of three other plants which contain glucosinolates.

RESULTS

The enzyme obtained from leaves of ***T. majus*** which catalyses the transfer of glucose from UDPG to PATH was purified by the procedure outlined in Table 1. The recovery of the enzyme, based on the fraction obtained after Sephadex G-50 chromatography, was 30 per cent and the purification was 20-fold. Attempts to purify the Sephadex G-50 protein fraction by DEAE chromatography were unsuccessful as the recovery of enzyme activity was only 2 per cent and little purification was achieved. The enzyme obtained after Sephadex G-100 chromatography could not be further purified by heating as it was inactivated when heated to 50" for 3 min. The specific activity of the Sephadex G-50 freeze-dried protein remained unchanged for more than 3 months when stored at -15". However, the enzyme purified by Sephadex G-100 chromatography was much less stable; the activity decreased 68 per cent in one day on freezing a solution of the protein and disappeared entirely on freeze-drying.

The amount of desulfobenzylglucosinolate formed from PATH and UDPG was found to be proportional to added enzyme and to time for incubation periods up to 20 min.

TABLE 1. PURIFICATION OF THE GLUCOSYLTRANSFERASE FROM ***T. majus***

Fraction	Volume * (ml)	Protein (mg/ml)	Recovery (%)	Specific activity (m unit†/ mg protein)	Purification
Sephadex G-50	20	6.1	100	2.3	1
25–80% $(NH_4)_2SO_4$	21	4.5	89	2.7	12
40–70% $(NH_4)_2SO_4$	3·0	8.7	75	8.1	3.5
Sephadex G-100	15	018	30	45.0	19.6

* The assay reaction mixture contained enzyme 20 μl, PATH 0·2 μmole, UDPG-^{14}C 0·2 μmole and buffer to a total volume of 100 μl.

† A m unit of enzyme activity is the amount of enzyme that catalyses the formation of one m μmole of product in 1 min at 30".

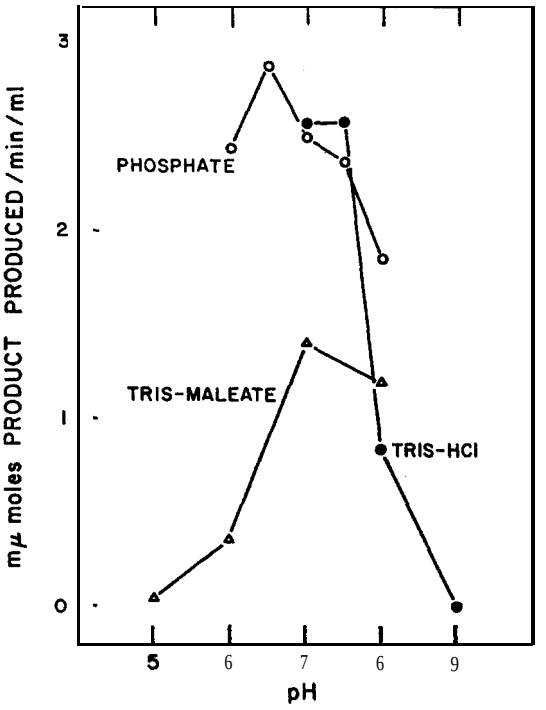

FIG. 2. THE EFFECT OF pH AND DIFFERENT BUFFERS ON THE GLUCOSYLTRANSFERASE. The reaction mixture contained in a total volume of 100 μl: enzyme 20 μl, PATH 0·2 μmole, UDPG-^{14}C 0·2 μmole. In each case the final concentration of the buffer was 60 mM containing 30 mM MCE.

The molecular weight of the enzyme (ca. 50,000) was determined by the method of Andrews[8] using a Sephadex G-100 column with bovine serum albumin and cytochrome C as standards for calibration. The pH optimum was between 6·5 and 7·5 (Fig. 2), but a greater activity was exhibited in phosphate and Tris-HCl buffers than in Tris-maleate. The pH optimum for PATH glucosylation is therefore similar to that found for many *O*-glucosylating enzymes obtained from higher plants (see Ref. 9).

The specificity of the transferase for the sugar nucleotide donor and for the carbohydrate transferred was investigated. The reaction conditions and results are listed in Table 2. ADPG, CDPG and GDPG were inactive as glucosyl donors. At the termination of incubation of these non-radioactive nucleotides with PATH, the solvent was removed under a stream of air and the residue was extracted into methanol. The methanol extract was concentrated and chromatographed in the usual manner. No UV absorbing spots which corresponded with desulfobenzylglucosinolate were detected. The enzyme could utilize both UDPG and TDPG as glucosyl donors, however, UDPG was ten times more active than TDPG. Two other uridine nucleotide sugars were tested to determine if another hexose or a pentose could be transferred to PATH. In each instance the entire reaction mixture was spotted on the chromatogram. No evidence for transfer of galactose-^{14}C or

[8] P. ANDREWS, *Biochem. J.* 91,222 (1964).
[9] K. HAHLBROCK and E. E. CONN, *J. Biol. Chem.* 245, 917 (1970).

TABLE 2. SPECIFICITY OF THE ENZYME FOR THE NUCLEOTIDE DONOR AND THE SUGAR TRANSFERRED

Nucleotide	Reaction conditions* Nucleotide (μmole)	PATH (μmole)	Enzyme (μl)	Total volume (ml)	Reaction time	Reaction (%)
ADPG	5	5	200	1	2 hr	0
CDPG	5	5	200	1	2 hr	0
GDPG	5	5	200	1	2 hr	0
TDPG-^{14}C	0·2	0·2	20	0·1	10 min	12.2
UDPG-^{14}C	0·2	0·2	20	0·1	10 min	100
UDP Galactose						
-^{14}C†	$1{\cdot}37 \times 10^{-4}$	5×10^{-4}	20	0·03	2 hr	0
	$1{\cdot}37 \times 10^{-4}$	5×10^{-3}	20	0.03	2 hr	0
UDP Xylose						
-^{14}C†	3.36×10^{-4}	5×10^{-4}	20	0·03	2 hr	0
	3.36×10^{-4}	5×10^{-3}	20	0.03	2 hr	0

* In each case the enzyme employed was derived from the 40–70% $(NH_4)_2SO_4$ fraction and the buffer was 0·01 M Tris-HCl pH 7·4 containing 5 mM MCE.

† The specific activity of UDP-galactose-^{14}C was 289 μCi/μmole and UDP-xylose-^{14}C was 173 μCi/μmole.

xylose-^{14}C to PATH was evident as the only radioactive areas on the developed chromatogram were near the origin and corresponded to the labeled substrates.

All of the thiohydroximate homologues examined, except acetothiohydroximate, were active as glucose acceptors (Table 3). The reason the enzyme could not utilize acetothiohydroximate remains unexplained but may be associated with a greater degree of instability of this substrate under the assay conditions. The oxygen analogue, phenylacetohydroximic acid, and the aliphatic alcohols and thiols listed in the lower part of Table 3 (all ionized at pH 7·4 to a lower degree than the thiohydroximates) were inactive as glucose acceptors. The compounds tested, although limited in number and type, could reasonably

TABLE 3. SPECIFICITY OF THE GLUCOSYLTRANSFERASE FOR THE GLUCOSE ACCEPTOR

Substrate*	Relative reaction rate (%)
Acetothiohydroximate	0
Propiothiohydroximate	50
Butyrothiohydroximate	70
Isobutyrothiohydroximate	41
4-Methylthiobutyrothiohydroximate	77
Benzothiohydroximate	10
Phenylacetothiohydroximate	100
Phenylacetohydroximic acid	0
Ethanol	0
β-Mercaptoethanol	0
Cysteine hydrochloride	0

* The reaction mixture contained enzyme 25 μl, substrate 0·5 μmole, UDPG-^{14}C 0·5 μmole and buffer to a total volume of 100 μl.

be expected to act as substrates for a non-specific glucosyltransferase. Our results indicate that the glucosylating enzyme possesses a degree of specificity for the thiohydroximate function.

Since PATH is a natural compound in the plant from which the enzyme was derived, it is not surprising that PATH proved to be the most active substrate tested. Glucosylation of the least active substrate, benzothiohydroximate, is of particular interest since no corresponding glucosinolate has yet been detected in plants.

The K_m values for PATH and UDPG were 0·65 and 1·54 mM respectively, as calculated from plots[10] of the data. The values for 4-methylthiobutyrothiohydroximate and UDPG were 1·05 and 1·47 mM respectively. The K_m values were obtained by keeping the concentration of either UDPG or the thiohydroximate constant at 5 mM while the concentration of the other substrate was varied between 0·2 mM and 5 mM.

The effect of a number of inhibitors on the enzymic transfer of glucose from UDPG to PATH was examined (Table 4). The enzyme was strongly inhibited by mercuric chloride and p-chloromercuribenzoate but was only moderately inhibited by *N*-ethylmaleimide. Potassium cyanide and iodoacetic acid were completely ineffective.

The activity of the enzyme was accelerated 39-66 per cent by the sulfhydryl compounds listed in Table 5 and 21-36 per cent by the chelating agents. The reducing agents, ascorbic acid and sodium hydrosulfite also were effective in increasing the enzymic activity. When the enzyme extract of *T. majus* was passed through a Sephadex G-25 column equilibrated with 5 mM Tris-HCl pH 7·4 but containing no β-mercaptoethanol, its activity disappeared completely and the enzyme activity could not be reactivated by the addition of any of the compounds listed in Table 5. Kleinhofs *et al.*[11] have reported an 0-glucosylating enzyme isolated from *Melilotus alba* which possessed an absolute requirement for sulfhydryl com-

TABLE 4. EFFECT OF INHIBITORS ON THE GLUCOSYLTRANSFERASE ACTIVITY

Inhibitor added*	Concentration (M)	Inhibition (%)
Potassium cyanide	10^{-3}	0
Iodoacetic acid	10^{-3}	0
Mercuric chloride	10^{-6}	17
	10^{-5}	36
	10^{-4}	96
p-Chloromercuribenzoate	10^{-6}	3
	10^{-5}	86
	10^{-4}	92
	10^{-3}	100
N-Ethylmaleimide	10^{-4}	0
	10^{-3}	9

* The reaction mixture contained, enzyme 50 μl, PATH 0·2 μmole, UDPG-^{14}C 0·2 μmole and 0·1 M Tris-HCl pH 7·4 to a total volume of 100 μl. The Sephadex G-100 derived enzyme fraction was dialysed for 1·5 hr prior to its use against 0·1 M Tris-HCl pH 7·4 buffer which did not contain MCE.

[10] H. LINEWEAVER and D. BURK, *J. Am. Chem. Soc. 56,658 (1934).*
[11] A. KLEINHOFS, F. A. HASKINS and H. J. GORZ, *Phytochem. 6, 1313 (1967).*

TABLE 5. EFFECT OF SULFHYDRYL, CHELATING AND REDUCING COMPOUNDS ON THE GLUCOSYLTRANSFERASEACZTNITY

Compound added*	Concentration (M)	Stimulation (%)
Cysteine hydrochloride	10^{-3}	41
	10^{-2}	66
Diothiothreitol	10^{-3}	42
	10^{-2}	69
Glutathione	10^{-3}	21
	10^{-2}	55
β-Mercaptoethanol	10^{-3}	6
	10^{-2}	39
Ethylenediaminetetra-acetic acid disodium salt	10^{-5}	18
	10^{-4}	35
	10^{-3}	36
Dipyridyl	10^{-4}	1
	10^{-3}	22
0-Phenanthroline	10^{-5}	0
	10^{-4}	26
	10^{-3}	21
Ascorbic acid	10-J	21
	10^{-2}	27
Sodium hydrosulfite	10^{-3}	8
	10^{-2}	19

* The preparation of the enzyme and the reaction conditions were the same as described in Table 4.

pounds; either β-mercaptoethanol or cysteine were required. However, requirement for sulfhydryl compounds is not characteristic of all plant *O*-glucosylating enzymes.[9] The stimulation of the *T. majus* enzymic reaction appears to be caused by masking the action of heavy metals (sulfhydryl compounds and chelating agents) and by protecting the enzyme from oxidation (reducing agents and sulfhydryl compounds).

The presence of a similar glucosyltransferase activity was investigated in three other glucosinolate-containing plants namely: A. *lapathifolia*, *S. alba* and N. *officinale*. The leaf protein extract of each species was passed through a Sephadex G-25 column and freeze-fried. Desulfobenzylglucosinolate was identified as the reaction product when each of the freeze-dried protein extracts was incubated with PATH and UDPG.

DISCUSSION

The transferase enzyme isolated from the leaves of *T. majus* was shown to catalyse the S-glucosylation of a number of thiohydroximates possessing both aliphatic and aromatic side chains with the exception of acetothiohydroximate. It should be noted that the occurrence of the glucosinolate corresponding to acetothiohydroximic acid seems to be almost entirely restricted to members of the family Capparidaceae.[12] Members of this plant family should be investigated for the occurrence of an acetothiohydroximate glucosylating enzyme and a study made of its specificity.

[12] M. G. ETTLINGER and A. KJAER. Sulfur compounds in plants. In *Recent Advances in Phytochemistry* (edited by T. J. MAWBRY, R. E. ALSTON and V. C. RUNECKLES), p. 59, Appleton-Century-Crofts, New York (1968).

The enzyme exhibited a high specificity for the nucleotide donor and of the nucleotides tested UDPG was the most active glucosyl donor. The enzyme failed to catalyze the transfer of galactose or xylose from UDP to PATH, a result consistent with the fact that all natural mustard oil glycosides are glucosidic. We previously suggested[5] the enzyme be named a UDP glucose: thiohydroximate glucosyltransferase and the present data is consistent with this suggestion.

The combined data from plant feeding experiments,' the identification of PATH as a natural compound in *T. majus*,[5] as well as the isolation of the glucosyltransferase, constitute convincing evidence that both phenylacetothiohydroximate and desulfobenzylglucosinolate are intermediates in the biosynthesis of benzylglucosinolate. The isolation of an enzyme from other glucosinolate-containing plants having a similar glucosyltransferase activity suggests that glucosylation of thiohydroximates to yield desulfoglucosinolates may be a reaction common to the formation of other, if not all, glucosinolates.

The mechanism of formation of thiohydroximic acids in higher plants has not been determined, although we have previously established that PATH is formed in *T. majus* from phenylacetaldoxime. Ettlinger and Kjaer[12] have suggested the *aci* tautomers of primary nitro compounds may be intermediates between aldoximes and thiohydroximic acids.

EXPERIMENTAL

Materials. ^{14}C-Labeled sugar nucleotides were purchased from commercial sources. The specific activities of UDPG and TDPG were adjusted by addition of carrier to 0·40 μCi/μmole.

The Na salt of phenylacetothiohydroximic acid and desulfobenzylglucosinolate were prepared previously.' The other thiohydroximic acids (each recovered as the Na salt) were synthesized by the method of Mizukami and Nagata[13] from the corresponding thionic acid methyl esters which, in turn, were obtained from the methyl imidate HCl-ides by the action of H_2S. The NMR spectrum of each of the thiohydroxamates formed were recorded using a Varian HA-100 spectrometer. The proton signals recorded for each compound were consistent with the expected structures. For example, the NMR spectrum of sodium propiothiohydroximate (d_6-DMSO), showed signals at δ 1.39 (triplet J = 7 Hz, CH_3), δ 2·54 (quartet J = 7 Hz, CH_2) and δ 9·4 (singlet, remaining thiohydroximic acid proton). Integration of the proton signals indicated the presence of a single proton δ 9-10 and this proton signal disappeared on addition of D_2O (compare NMR spectra of thiohydroximic acids recorded by Nagata and Mizukami[3]).

Desulfomethylglucosinolate was obtained by pyridine desulfation of methylglucosinolate.[14] Desulfo-3-methylthiopropylglucosinolate was synthesized from Na 4-methylthiobutyrothiohydroxamate (70 mg) and acetobromoglucose (155 mg) dissolved in 2 ml of a mixture of MeOH and acetone (1:3, v/v) and kept at room temp. for 3 hr. The solution was filtered to remove the precipitate and concentrated *in vucuo.* The residue, dissolved in 5 ml of MeOH saturated with NH_3, was allowed to stand at 0" overnight. After filtration and concentration *in vacuo*, the mixture was put on a column (2.7 x 30 cm) of silicic acid and eluted with a mixture of MeOH and $CHCl_3$ (1: 5, v/v). The fraction containing the desulfoglucosinolate appeared after 420 ml of the solvent was eluted. This fraction (about 100 ml) was pooled and concentrated *in vacuo* and passed through a small column containing 0·5 g of Dowex 1 x 8 (OH-) ion exchange resin. The desulfoglucosinolate was eluted using a linear gradient of NaCl (0–0·1 M). The desulfoglucosinolate fraction obtained was neutralized with dil. HCl and concentrated *in vucuo. The* residue was extracted with MeOH. After evaporation of MeOH, 37 rng of pure desulfo-3-methylthiopropylglucosinolate was obtained (yield, 30 %).

Enzyme Purification

Sephudex G-50. Leaves of *T. majus*, *S. alba*, *N. officinale* and *A. lapathifolia (SO g* fr. wt.) were cut with scissors and ground in a chilled mortar with washed sand and 50 ml of 0·1 M Tris-HCl pH 7·4 containing 0·05 M MCE and 1 g of Polyclar AT. The mixture was squeezed through nylon mesh and centrifuged at 32,000 g for 30 min at 0°. The supematant from the *T. majus* extract was passed through a Sephadex G-50

[13] S. MIZUKAMI and K. NAGATA, *Chem. Phurm. Bull. Tokyo 14, 1249 (1966).*
[14] M. G. ETTLINGER and G. P. DATEO, JR., Studies of Mustard Oil Glucosides, Final Report Contract DA 19-129-QM-1059, U.S. Army Natick Laboratories, Natick, Massachusetts (1961).

column, the other 3 extracts through a Sephadex G-25 column. Both columns were equilibrated and developed with 5 mM Tris-HCl pH 7·4 containing 0·5 mM MCE. The protein eluate was freeze-dried.

Ammonium sulfate fractionation. The freeze-dried powder of *T. majus* was dissolved in 0·1 M Tris-HCl pH 7·4 containing ~O.05 M MCE (100 mg/10 ml). The proteins in solution were fractionated by the addition of solid $(NH_4)_2SO_4$. The fraction precipitating between 0–25% $(NH_4)_2SO_4$ saturation was discarded and the fraction (25–80% saturation) was dissolved in 20 ml of 0·1 M Tris-HCl pH 7·4 containing 0·05 M MCE and dialysed for 1·5 hr against the same buffer. From the dialysed protein solution a 40–70% $(NH_4)_2SO_4$ saturation fraction was obtained and dissolved in 3 ml of 10 mM Tris-HCl pH 7·4 containing 5 mM MCE.

Sephadex G-100 column chromatography. The 40–70% $(NH_4)_2SO_4$ fraction was passed through a Sephadex G-100 column (2.5 x 45 cm) equilibrated and developed with the same buffer in which the fraction was dissolved, but containing 0·1 M NaCl. A flow rate of 12 ml/hr was maintained. The enzyme activity was maximum when about 120 ml had been eluted and the activity was recovered in a total of 15 ml. Table 1 summarizes the purification procedure.

Enzyme Assays

The assay for glucosyltransferase activity was based on the measurement of radioactivity incorporated into the reaction product from ^{14}C-labeled sugar nucleotides. The reaction conditions are given in the footnotes to the Figures and Tables, and, except where otherwise noted, the enzyme employed was the protein fraction obtained after Sephadex G-100 chromatography and the buffer was 0·1 M Tris-HCl pH 7·4 containing 0·05 M MCE. The enzyme reactions were carried out in small tubes at 30" for 10 min. The reaction was stopped by the addition of 2 vol. of MeOH. The supernatant was passed through a small column containing 300 mg of anionotropic alumina of activity grade 1 (M. Woelm, Eschwege, Germany). This column absorbed the substrates of the enzyme reaction, both the sugar nucleotides and the thiohydroximates, but did not retain the desulfoglucosinolates. The column was washed with 1·5 ml MeOH. The washings were added to the eluate and the radioactivity in the eluate was determined by liquid scintillation counting. One unit of enzyme activity is defined as the amount of enzyme that catalyses the formation of one μmole of product in 1 min at 30".

The enzyme reaction product obtained from PATH and UDPG was identified as desulfobenzylglucosinolate by paper chromatography, TLC and GLC as previously described.[5] The products obtained in experiments relating to the specificity of the enzyme for the glucose acceptor (Table 3) and nucleotide donor (Table 2) were qualitatively examined by chromatography on Whatman No. 1 paper which was developed overnight using *n*-BuOH–HOAc–H_2O (4:1:1·8 by vol.). Desulfoglucosinolates were detected as UV absorbing (short wave) areas. Radioactive areas on the paper were detected using a Nuclear Chicago Chromatoscanner. The R_f values of the compounds formed enzymatically from UDPG-^{14}C and PATH or 4-methylthiobutyrothiohydroximate were found to be identical with synthesized desulfobenzylglucosinolate (0.60) and desulfo-3-methylthiopropylglucosinolate (0.51). The products obtained when other thiohydroximates were incubated in the presence of UDPG-^{14}C and the glucosyltransferase were judged to be the corresponding desulfoglucosinolates on the basis that they were not absorbed on the alumina column and they gave only one radioactive spot on the paper chromatogram in addition to UDPG-^{14}C. The R_f values of these desulfoglucosinolates were: ethyl- (0·37), propyl- (0·48), isopropyl- (0·50) and phenyl- (0·55). The R_f of desulfomethylglucosinolate was 0·3 1.

Protein Assay

Protein was determined by the method of Lowry *et al.*[15]

Acknowledgements-The authors thank Dr. L. R. Wetter and T. Oka for their advice and discussions throughout this work and Mr. M. D. Chisholm for the gift of phenylacetohydroxamic acid. They also thank Mr. M. Mazurek for the NMR spectra.

[15] 0. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem. 193,265 (1951).*