Isothiocyanates. XXXVII. A new method of determination of natural and synthetic isothiocyanates and glucosinolates

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A spectrophotometric method for the determination of isothiocyanates has been worked out. It is based on the reaction of isothiocyanates with thioglycolic acid by which they are quantitatively converted into *N*-substituted thiocarbamoylmercaptoacetates or 3-substituted rhodanines having characteristic u.v. absorption maxima. Glucosinolates in plant tissues may thus be determined after their extraction and enzymic hydrolysis by determining the liberated isothiocyanates. A microsynthesis, separation by paper and thin-layer chromatography, different modes of detection and some physicochemical properties of 3-substituted rhodanines are also described.

Mustard glucosides (glucosinolates GS) are a rather well characterized group of naturally occurring substances. They are found in plants of certain botanic family. Some fifty mustard glucosides containing invariably an α-D-glucopyranose residue esterified with sulfuric acid have been described in the literature. By enzymic hydrolysis with thioglucosidase (thioglycoside glucohydrolase EC 3.2.3.1.) glucosinolates yield, by intramolecular rearrangement, D-glucose, sulfate, and the corresponding mustard oil.

In a similar manner glucosinolates give isothiocyanates (ITC) by chemical hydrolysis. Most of the naturally occurring ITC have been prepared by synthesis; the synthesis of some 500 other synthetic ITC having various structural features have been described [1].

The reaction of ITC with thioglycolic acid (TA) offers new possibility of quantitative determination of ITC, the former being also useful in the study of the kinetics of the formation of ITC as well as the products of decomposition of GS and synthetic producers of ITC. Based on previous studies of the kinetics and mechanism of the reaction of ITC with nucleophiles, including TA [2–4], the synthesis, i.r. and u.v. spectra of 3-substituted rhodanines (R) [6, 7], the products of the reactions of ITC with thioglycolate [5] a method has been developed for spectrophotometric determination of ITC by their conversion into thiocarbamoylmercaptoacetates (TCMA) or R. A modified microsynthesis of R, solvents for chromatographic separation by paper chromatography and thin-layer chromatography on silicic acid, several modes of detection and basic physicochemical data of R, including their molar solubilities in water and some organic solvents, distribution coefficients for the system octanol–water, u.v. spectra of R and TCMA as well as kinetic constants of the addition reaction of ITC with TA and cyclization reaction of TCMA, are also described.

Experimental

The rhodanines under investigation, listed in Table 1, were prepared as described in [5]. Modifying the known [6] microsynthesis some R derived from rare naturally occurring ITC were prepared in the following manner.

Table 1

Characterization of 3-substituted rhodanines

No.	Substituent	M.p., B.p. °C/torr	S 10^{-5} [mol l^{-1}]	$3 + \log P$	λ_{max} [nm] log ε	λ_{max} [nm] log ε
I	Methyl	69–70	156.1	1.15	258 4.23	294 4.29
II	Ethyl	128/4	223.1	1.65	260 4.19	294 4.27
III	*n*-Butyl	145/4	138.9	2.65	260 4.31	296 4.39
IV	Allyl	46	—	—	260 4.03	296 4.13
V	Benzyl	82.5–83	2.51	3.34	260 4.23	296 4.29
VI	4-Bromobenzyl	95–96	0.25	4.36	260 4.30	292 4.32
VII	β-Phenylethyl	106.5–107	0.84	3.78	262 4.11	296 4.18
VIII	Phenyl	112–114	1.39	2.78	258 4.13	296 4.27
IX	4-Dimethylaminophenyl	204–206	0.60	2.69	266 4.41	294 4.23
X	4-Tolyl	166.5–168	0.84	3.30	256 4.06	296 4.28
XI	4-Nitrophenyl	226–228	0.73	2.02	250 4.27	294 4.29
XII	4-Ethoxyphenyl	185–188	0.47	3.24	250 4.21	296 4.37

S — water solubility at 25°C; P — distribution coefficient in the system octanol—water at 25°C.

ITC (0.25 mmole) was dissolved in a mixture of acetone (5 ml) and triethylamine buffer (pH 10,5 ml) containing TA (0.21 mmole). The mixture was kept at 40°C for 1 hour, the solvents were removed on a rotary evaporator and the residue was dissolved in acetic acid (2.5 ml) saturated with hydrogen chloride. Rhodanines obtained from these solutions by chromatographic purification were used as standards for qualitative chromatographic and spectrophotometric determination of the corresponding rhodanines.

The derivatives were characterized by u.v. spectral data (in methanol containing 0.1% acetic acid for stabilization), by their solubility in water (containing 0.1% acetic acid) and in organic solvents (ethyl acetate, cyclohexane, and *n*-hexane) and by distribution coefficients in the system octanol—water (containing 0.1 N-HCl). The solubility was determined as described in [8].

The distribution coefficients of 3-methyl-R and 3-benzyl-R were determined spectrophotometrically in the system 0.1 N hydrochloric acid—octanol at 25°C. The acidic medium is necessary to stabilize the rhodanines. The concentration of substances was determined in the octanol phase. The given values of log P (for 3-methyl- and 3-benzyl-R) are average values of three runs. Based on the log P and S values of R and using the π constants of the substituents ($\pi = \log P_x - \log P_H$) [9, 10] the values log P for other R were calculated. These served for estimate of the lipophility of these substances.

Quantitative monitoring of the reaction of isothiocyanates with thioglycolate

Procedure A

A mixture of ITC (0.5×10^{-3}—3×10^{-3} M) in acetone (0.1 ml), borate buffer (2.4 ml, 0.1 M, pH 10) and TA (10^{-3} M) in a 10-ml test tube (closed with a glass ground joint stopper) was kept at room temperature for 1 hour. Hydrochloric acid (4 N, 0.5 ml) was added followed, after 4 hours at room temperature (or 2 hours at 40°C), by the addition of ethyl acetate (5 ml). The mixture was shaken intensively and after 5 minutes a portion of the upper layer was withdrawn for spectrophotometric determination of the concentration of R. A blank experiment (a mixture of buffer with ethyl acetate) was run simultaneously.

Procedure B

To a mixture of McIllvain buffer (9 ml, 0.1 M, pH 7.5) and a freshly made water solution of TA (0.5 ml, 10^{-1} M) methanolic ITC (0.5 ml, 10^{-3} M) was added and the solution was kept at 25 ± 0.1°C for 60 minutes. The u.v. spectrum of the reaction mixture (rhodanine) was taken against a blank experiment containing no ITC.

Procedure C

A freshly made water solution of TA (0.5 ml, 10^{-1} M) and methanolic ITC (0.5 ml, 10^{-3} M) was added to borate buffer (9 ml, 0.1 M, pH 10) and after 10 minutes at 25 ± ± 0.1°C the u.v. spectrum of the reaction mixture was taken against a blank containing no ITC (indication of the concentration of the corresponding N-substituted thiocarbamoylmercaptoacetate).

Determination of GS in plant tissues

The isolation of GS (from the seeds of *Tropaeolum majus*, *Putranjiva Roxburghii*, and *Brassica nigra*) and their enzymic cleavage was carried out according to the described procedure [7] modified as follows: Freshly made seed dust (1 g) was extracted with 70% methanol (10 ml) on a water bath. The mixture was filtered and the extract was concentrated under diminished pressure at 40°C. Water (0.5 ml) was added and the solution was again evaporated. Distilled water (4 ml) was added to the residue followed by the addition of 1 M citrate buffer (0.2 ml, pH 6.5), ascorbic acid (5 mg), a solution of the enzyme myrosinase (0.8 ml, corresponding to 0.15 mg of protein) and the solution was kept at 37°C for 2 hours. Acetone (2 ml), borate buffer (pH 10, 3 ml), and TA (120 mmoles) was added, the mixture was shaken and kept at 40°C for 2 hours. Hydrochloric acid (4 N, 1 ml) was added, the solution was kept at 40°C for 2 hours and after having been cooled the solution was extracted with ethyl acetate (10 ml). The clear solution was evaporated under reduced pressure at 70°C and the residue was dissolved in acetic acid (1 ml). The solution

thus obtained was used for qualitative or quantitative determination of R directly (spectrophotometrically), or after separation by chromatography (elution of R with ethanol). U.v. spectra of alkyl- and arylrhodanines show two characteristic absorption bands at λ_{max} ~260 and ~295 nm (see also [5]). For routine analysis it is sufficient to take the spectrum at 296 nm.

Chromatographic separation

The following conditions were used: *1.* *n*-butanol—ethanol—water = 4 : 1 : 4, Whatman No. 1 paper, descending technique, 5 hours (front 17 cm); *2.* chloroform saturated with water, Whatman No. 1 paper, descending technique, 2 hours (front 38 cm); *3.* carbon tetrachloride containing 2% of acetic acid, descending technique, 4 1/2 hours (front 30 cm) [11]; *4.* *n*-heptane—ethylene chloride—75% formic acid = 12 : 6 : 1, Whatman No. 1 paper, descending technique, *ca.* 4 1/2 hours (front 34 cm) [12]; *5.* benzene—ether = 9 1. Silica gel G [5].

Chromatography was performed at 23 ± 0.5°C.

The sensitivity of the detection of R, the corresponding ITC and other possible sulfur-containing compounds (thiourea, TA, TCMA) was evaluated by applying 1 μl of water or alcoholic solutions (in the case of R, in order to stabilize the compounds, acetic acid was added to the used solutions) in the concentration range of 0.025—25 mmoles.

Detection: iodine-azide reagent combined with spraying of the chromatogram with 1% starch solution [12], ammonium silver nitrate (200 mg of silver nitrate in 5 ml of concentrated ammonia made up to 100 ml with methanol), Grote's reagent [13]. The presence/absence of compounds not detected with the above-mentioned reagents was ascertained by determining the u.v. absorption at 250 and 350 nm and/or by illumination with an u.v. lamp through a plexiglass board coated with cadmium borate. The detection on Silica gel G was by spraying with ammonium silver nitrate.

Results and discussion

Synthesis on a micro-scale, u.v. spectra, distribution coefficients of rhodanines

The physical constants and other relevant data of 3-substituted R derived from common ITC prepared on a large scale are summarized in Table 1. Methyl-, propyl-, allyl-, and benzylrhodanines were prepared also by the micro-scale procedure. Rhodanines derived from rare naturally occurring ITC were prepared only by the microsynthesis.

Table 2

Solubilities of some 3-substituted rhodanines in organic solvents at 25°C
($S \cdot 10^2$ [mol l^{-1}])

No.	Ethyl acetate	Cyclohexane	*n*-Hexane
XI	5.7	—	—
VIII	7.4	—	
VI	70.0	1.4	0.6
V	54.0	0.9	0.6
I	174.0	1.6	1.8

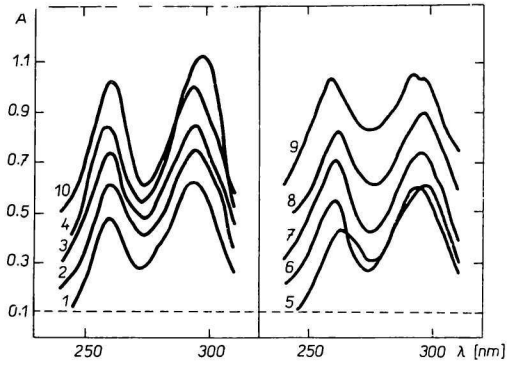

Fig. 1. Ultraviolet absorption spectra of 3-substituted rhodanines derived from ITC prepared by microsynthesis. Concentration of rhodanines 4×10^{-5} M in absolute ethanol containing 0.05% of glacial acetic acid. The individual curves, excepting those of the first two derivatives (1-methyl- and 5-isobutylrhodanines) are shifted by an absorbancy value of 0.1.

1. methyl-; *2.* *n*-propyl-; *3.* isopropyl-; *4.* allyl-; *5.* isobutyl-; *6.* (3-methylsulfonylpropyl)-; *7.* (4-methylsulfonylbutyl)-; *8.* 5-methylthiopentyl-; *9.* *o*-methoxybenzyl-; *10.* benzylrhodanines.

Previous information [6] and the molar absorption bands indicated that under the micro-scale conditions the conversions of ITC into the corresponding rhodanines were better than 95%. The same can be concluded from the u.v. spectra of R (Fig. 1) derived from rare ITC. In view of the small amount of the material available these new compounds were not characterized by physicochemical constants.

As to the distribution coefficients P it should be emphasized that only in the case of two derivatives (methyl- and benzylrhodanines) the given values were obtained by direct measurements. The other P values (Table 1) were obtained by calculation, taking into account π constants of the substituents and, hence, these should serve only for rough estimate of P for the system water—octanol or other systems. Although the values obtained by direct measurements are surprisingly low these are adequate, considering the assumed ionic nature of these substances. The solubility data of selected R in some solvents are summarized in Table 2.

Chromatography of rhodanines

Four different solvent systems for paper chromatography were examined, their efficiency to separate different R as well as their separation from ITC, TCMA, GS, and other substances were judged. The systems *1* and *3* are commonly used for separation of mustard GS, system *2* for separation of substances related to thiourea, and system *4* for the separation of 2-thiohydantoins. The found R_F values are in Table 3. For quantitative determination of R these can be, after chromatographic separation, extracted with ethyl acetate or ethanol, and the concentration may then be determined spectrophotometrically. 3-Substituted R can be excellently separated on Silica gel G.

The detection of R on paper chromatograms can be conveniently done with the iodine-azide reagent combined with the spraying with 1% starch solution. 5×10^{-6} μM of

Table 3

R_F values of 3-substituted rhodanines and some related compounds*

Compound	R_F Values		
	Solvent *A*	Solvent *C*	Solvent *D*
Rhodanines			
Methyl	0.91	0.91	0.73
n-Propyl	1.00	1.00	0.85
Allyl	0.93	0.98	0.79
3-Methylsulfonylpropyl	0.78	0.11	0.03
4-Methylsulfonylbutyl	0.79	0.20	0.03
5-Methylthiopentyl	1.00	1.00	0.88
Benzyl	0.94	0.91	0.86
β-Phenylethyl	0.91	0.89	0.83
Phenyl	0.83	0.40	0.70
Phenylthiourea	0.82	0.18	0.04
Methylthiourea	0.59	—	—
N-Phenylthiocarbamoylmercaptoacetate	0.60	—	—
N-Benzylthiocarbamoylmercaptoacetate	—	—	0.10
Thioglycolic acid	0.16	0.10	0.00

* In solvent *B* most of the compounds move with the front.

R or 12.5×10^{-6} μM of TCMA can be detected with this reagent as yellow spots. Ammonium silver nitrate gives orange spots when 1 μl of 125 μM solution is applied whereas Grote's reagent required more concentrated solution. Rhodanines show more pronounced u.v. absorption at 250 nm than at 350 nm. The spots are visible by applying 1 μl of 125 μM solution. The u.v. detection of 3-alkyl- and 3-arylrhodanines becomes more sensitive (by a factor of 5—10) when the chromatograms are illuminated through a plexiglass board coated with cadmium borate.

Determination of isothiocyanates

Taking into account our previous studies [2—5, 14] on quantitative reactions of ITC with nucleophiles it seemed advantageous to apply the results obtained for the determination of TA. ITC react with the —SH groups of TA after ionization much faster than with other nucleophiles (*e.g.* with amines or hydroxides), to give TCMA. These products,

Table 4

Rate constants of the addition and cyclization reaction of basic isothiocyanates

ITC	$k_{ad\ SH}$ [$min^{-1}\ mol^{-1}\ l$]	$t_{1/2}$ [min]	k'_{cycl} [min^{-1}]	$t_{1/2}$ [min]	$k_{ad\ OH}$ [$min^{-1}\ mol^{-1}\ l$]
Benzyl	31.9×10^3	0.043	4.83×10^{-3}	143.4	5.95
Phenyl	63.0×10^3	0.022	3.50×10^{-3}	198.0	7.60
4-Bromophenyl	11.6×10^4	0.012	1.38×10^{-3}	502.1	—

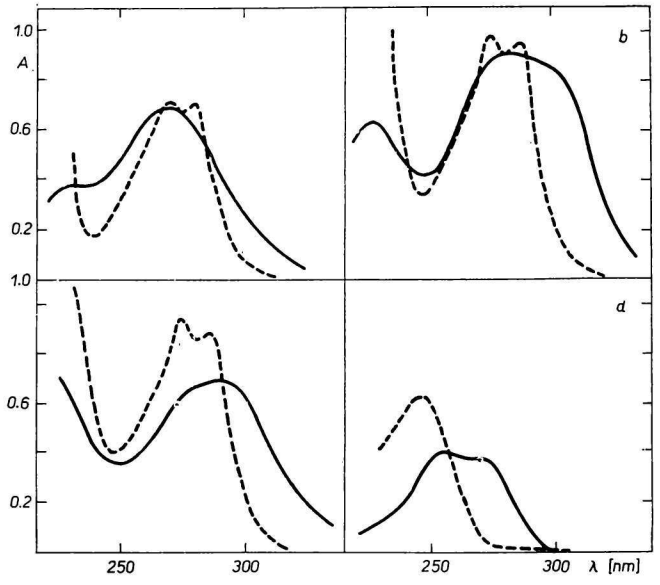

Fig. 2. Ultraviolet absorption spectra of the reaction mixtures of ITC with TA in a borate buffer (0.1 M, pH 9.8). Reaction time 10 minutes, $t = 25°C$, starting concentration: ITC 5×10^{-5} M, TA 10^{-3} M.
——— reaction products (TCMA) formed from the following ITC: *a*) phenyl, *b*) 4-bromophenyl, *c*) 4-methoxyphenyl, *d*) benzyl derivatives; — — — — the corresponding ITC ($c = 5 \times 10^{-5}$ M) in methanol.

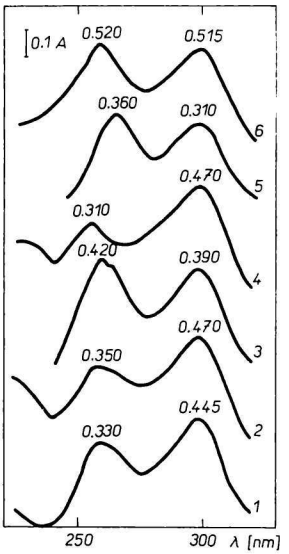

Fig. 3. Ultraviolet absorption spectra of the reaction mixture of ITC with TA in McIllvain buffer (0.1 M, pH 7.5). Reaction time 10 minutes, $t = 25°C$, starting concentration: ITC 3.13×10^{-5} M, TA 1.25×10^{-3} M. Measurement run against a blank without ITC. The individual curves correspond to the reaction products from the following ITC added:
1. phenyl-; *2.* 4-bromophenyl-; *3.* benzyl-; *4.* 4-methoxyphenyl-; *5.* *n*-butyl-; *6.* 4-acetylphenyl derivatives.

when made acidic, cyclize to yield the corresponding 3-substituted R. The mechanism of the reaction of ITC with TA presumes dissociation with the formation of the reactive S-form. When the concentration of S^- is higher than that of ITC (depending upon the pH of the reaction medium) the reaction is quantitative in few minutes. Thus, under the reaction conditions of Procedure A (see Experimental) ITC are quantitatively converted into TCMA. When the reaction mixture is acidified the products are quantitatively cyclized to give the corresponding R which are extracted and determined spectrophotometrically. The conditions given in Procedure A take into account the low reactivity of alkyl ITC. The velocity constants log k of the addition and cyclization reaction of three basic types of ITC are given in Table 4. These are taken from [14] where these types of reactions were studied in detail.

ITC can be also determined using Procedure *C i.e.* by conversion into TCMA. Although the products cannot be isolated from the reaction mixtures, their presence can be ascertained by characteristic u.v. absorption spectra (Fig. 2).

When the reaction of ITC with TA is carried out at pH 7.5 (Procedure *B*) an addition reaction of ITC with TA takes place followed by immediate cyclization to give the corresponding R (Fig. 3). Depending upon the structure of ITC (mainly in the case of aromatic ITC) opening of the rhodanine ring with the formation of original addition products may occur. This happens probably due to the disturbed, otherwise complicated, equilibrium between the reaction products and various forms of TA (as a result of its oxidation). Therefore, Procedure *B* is suitable only for the determination of ITC.

Determination of GS

Determination of GS in plant tissues usually involves their extraction, enzymic hydrolysis followed by their conversion into corresponding derivative of thiourea subsequently determined gravimetrically or by titration methods. Alternatively, ITC can be converted into *N*-monosubstituted thiourea derivatives and determined by chromatography [15]. Owing to a great number of GS and hence the corresponding ITC isolated from plants (in some plants up to 3 GS are present) the identification of these substances requires several separation systems. Since these substances are alkyl or aralkyl derivatives the derivatives of thiourea derived from naturally occurring ITC do not show pronounced u.v. absorption. On the other hand TCMA and R have characteristic u.v. absorption and, thus, by the reaction of ITC with TA these products can be advantageously determined spectrophotometrically. The results of the determination of GS in the seeds of *Tropaeolum*

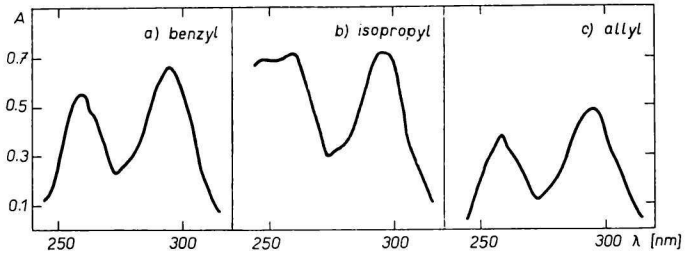

Fig. 4. Ultraviolet absorption spectra of rhodanines prepared from ITC isolated from the seeds of *Tropaeolum majus* (*a*), *Putranjiva Roxburghii* (*b*), and *Brassica nigra* (*c*).

majus, *Putranjiva Roxburghii*, and *Brassica nigra* (Fig. 4) are examples of such determination. The seeds of these species contain each one GS, namely glucotropeoline (benzyl ITC), glucoputranjivine (isopropyl ITC) and sinigrine (allyl ITC), respectively.

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