Biosynthesis of Indole Glucosinolates in Rape Seedlings under the Influence of some Sulphur Metabolism Inhibitors

In the plants of the family Brassicaceae a new type was found of biologically important indole glucosinolate – glucobrassicin (I)[1] and later also its N^1-methoxy-derivative – neoglucobrassicin (I)[2,3]. The biogenesis of glucobrassicin is elucidated only partially[4–6]. The indolylacetonitrile moiety of the glucobrassicin molecule originates from tryptophan, which is incorporated into glucobrassicin after decarboxylation[4]. The origin of the thioglucose moiety of glucobrassicin is not known as yet in detail[7–10]. Sulphur in glucobrassicin originates mainly from sulphate[11], sugar-bound sulphur can originate from cysteine[7] or methionine[12]. In the course of the study of indole glucosinolates biosynthesis, we examined the influence of some physiologically active substances, effecting sulphur metabolism (alloxan, molybdate, manganese) on the formation of glucobrassicin and neoglucobrassicin in rape seedlings. Alloxan does effect the metabolism of thiol compounds[13], by reaction with their –SH groups[14], molybdate inhibits sulphate reduction[15] and divalent manganese ions are inhibitors of the incorporation of sulphate-sulphur into organic sulphur-containing compounds[16].

We studied the influence of alloxan and molybdate (Na_2MoO_4 resp. K_2MoO_4) in the concentrations 10^{-2}, 10^{-3}, 10^{-4} and $10^{-5}M$ on the glucobrassicin and neoglucobrassicin content in etiolated rape seedlings after 1, 2, 3, 4 and 7 days of germination in the appropriate solutions on the one hand, and the effect of the same substances but 10^{-2}, 10^{-3} and $10^{-4}M$ and $MnCl_2$ 10^{-3} and $10^{-5}M$ on the incorporation of ^{35}S from $Na_2{}^{35}SO_4$ into glucobrassicin and neoglucobrassicin in hypocotyl segments from 8-day-old etiolated rape seedlings in vitro on other hand. In the cultivation experiment, seeds of rape (*Brassica napus* var. arvensis Lam.) were germinated on filter paper in the appropriate solutions in plastic boxes in a thermostat (25 °C) in the dark. After the appropriate time of germination, the seedlings were harvested and glucobrassicin together with neoglucobrassicin extracted from the fresh plant material with boiling methanol (10 g with 100 ml) twice, the combined extracts concentrated in vaccuo at 40 °C and aliquot portions spotted on chromatographic paper Whatman No. 1. The chro-

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^{35}S-activity of methanolic extracts and of individual glucosinolates glucobrassicin (Glubr.) and neoglucobrassicin (Neoglubr.) calculated for 100 hypocotyl segments

Experiment	Concentration (*M*)	^{35}S-activity of the methanol-soluble fraction (cpm)[a]	^{35}S-activity of methanol-soluble fraction (% of control)	^{35}S-activity of individual glucosinolates (cpm): average values with their mean deviations[b]		^{35}S-activity of individual glucosinolates (% of control)		^{35}S-activity of individual glucosinolates/ activity of methanol-soluble fraction (%)	
				Glubr.	Neoglubr.	Glubr.	Neoglubr.	Glubr.	Neoglubr.
Control	–	3,031,500	100	667,261 ± 64,094 *N* 6	398,078 ± 36,247 *N* 6	100	100	22.0	13.1
Alloxan	10^{-2}	1,922,300	63.6	197,100 ± 43,466 *N* 5	176,591 ± 34,892 *N* 5	29.5[e]	44.3[e]	10.2	9.1
	10^{-3}	2,021,000	66.8	365,066 ± 47,352 *N* 6	281,709 ± 16,660 *N* 6	54.7[d]	70.7[c]	18.1	13.9
	10^{-4}	2,209,000	72.8	154,900 ± 6,077 *N* 5	176,276 ± 13,143 *N* 5	23.2[e]	44.2[e]	7.0	7.9
K_2MoO_4	10^{-2}	3,125,500	103.0	50,619 ± 5,372 *N* 6	34,282 ± 8,762 *N* 6	7.6[e]	8.6[e]	1.6	1.1
	10^{-3}	1,880,000	62.2	231,428 ± 9,236 *N* 6	221,999 ± 41,452 *N* 5	34.6[e]	55.7[d]	12.3	11.8
	10^{-4}	2,773,000	91.6	239,176 ± 18,310 *N* 6	221,611 ± 12,773 *N* 6	35.8[e]	55.6[e]	8.6	7.9
$MnCl_2$	10^{-3}	8,566,521	282.6	1,231,792 ± 83,060 *N* 6	1,127,647 ± 49,363 *N* 5	184.6[e]	283.2[e]	14.4	13.1
	10^{-5}	4,371,000	144.2	561,201 ± 72,792 *N* 5	589,953 ± 103,650 *N* 5	84.1	148.2[c]	12.8	13.5

[a] Average value of 2 estimations. [b] *N* = number of estimations. [c] *P* 0.05. [d] *P* 0.01. [e] *P* 0.001.

matographic analysis was performed in the previously published way[17]. The glucobrassicin and neoglucobrassicin content in the water eluates from the paper chromatograms was estimated fluorometrically[17,18]. Each estimation was performed in 8–12 parallel replicates. In the experiment in vitro, 15 mm long segments from hypocotyls of 8-day-old etiolated rape seedlings were placed on filter paper discs in small Petri dishes (30 segments in the dish), then added 4 ml of the test solution (or distilled water in control experiment) and 1 ml $Na_2{}^{35}SO_4$ solution (1 mg $Na_2{}^{35}SO_4$, activity 15 μc). For any solution tested, 2 dishes were used. Hypocotyl segments were incubated 5 h in the dark, then washed free from labelled sulphate, placed on filter paper wetted with distilled water in new Petri dishes and left 19 h in the dark at 25 °C. Indole glucosinolates were extracted from the segments with boiling methanol and analysed by means of paper chromatography in the *n*-butanol-acetic acid-water mixture (4:1:2). The ^{35}S-activity on the chromatograms was detected with the apparatus 'Tesla automat' with use of 4 π-methane probe (range $3\times2/3\times4$). 10^2 cpm, 1600 mm/h shift, 2 mm slit, D = 10 sec). The glucobrassicin and neoglucobrassicin bands were cut out from the chromatograms, eluted twice with 50% hot methanol and in aliquots of the combined eluates ^{35}S-activity was estimated by liquid scintillation counting with use of the liquid scintillator LSD 31 (product of 'Spolana', National Enterprise, Neratovice, ČSSR) on the scintillation counter 'Nuclear Chicago Mark I'. There have been made 5–6 parallel estimations and the results tested statistically (*t*-test).

In the cultivation experiment, alloxan 10^{-2} and $10^{-4}M$, especially in the 2-day-old germinating seeds, caused an inhibition of the glucobrassicin and neoglucobrassicin formation. The decrease of their content in the dry weight was 30–48% ($P<0.001$). In the older seedlings (4th and 7th day), alloxan did not effect the content of glucobrassicin, but caused serious inhibition of neoglucobrassicin formation: the decrease was 50–84% ($P<0.05$) in all concentrations studied. Na_2MoO_4 $10^{-2}M$ did not significantly change the content of the 2 glucosinolates. $10^{-3}M$ concentration caused in the 4-day-old rape seedlings a great increase of glucobrassicin content (+385%, $P<0.001$), and also an increase of neoglucobrassicin content (+60%, $P<0.01$). In younger or older seedlings, $10^{-3}M$ Na_2MoO_4 had only an insignificant effect. The $10^{-4}M$ concentration decreased in the 1 day germinating seeds the glucobrassicin content (−59%, $P<0.001$), then the inhibitory effect continuously weakened, while in the 4-day-old seedlings the content of glucobrassicin increased on the contrary, very strongly (+285%, $P<0.001$). The content of neoglucobrassicin was not significantly effected. $10^{-4}M$ Na_2MoO_4 at the 7th day had only a weak, insignificant inhibitory effect on the glucobrassicin content, whereas the neoglucobrassicin content was significantly decreased (−75%, $P<0.001$). $10^{-5}M$ Na_2MoO_4 decreased in the 1-day-old germinating seeds the glucobrassicin content (−59%, $P<0.001$), then this effect weakened and began to appear stimulating action: in 4-day-old seedlings the glucobrassicin content increased very strongly (+400%, $P<0.001$), whereas the neoglucobrassicin content was not significantly altered. In the 7-day-old seedlings $10^{-5}M$ Na_2MnO_4 did not significantly effect the content of both indole glucosinolates.

The results of the experiment with hypocotyl segments are summarized in the Table. Alloxan in all 3 concentrations inhibited not only the sulphate uptake (probably due to changes in the membrane permeability as a consequence of reaction of alloxan with –SH groups in the membrane), but also the incorporation of ^{35}S into both glucosinolates, probably due to interference with thiol-compounds metabolism. K_2MoO_4, with exception of $10^{-2}M$ concentration, decreased the amount of methanol-soluble ^{35}S and in all concentrations used decreased also the incorporation of ^{35}S into both glucosinolates (Table) $MnCl_2$ evidently increased membrane permeability and formation of neoglucobrassicin, whereas formation of glucobrassicin was more weakly effected.

From comparison of the results achieved with germinating seeds and hypocotyl segments, it follows that alloxan in both cases exerted mainly an inhibiting effect on the indole glucosinolates biosynthesis. Molybdate in younger intact seedlings stimulated the formation of both glucosinolates, especially on the 4th day; later its effect was mainly insignificant. In hypocotyl segments molybdate, in all concentrations used, decreased formation of both glucosinolates. Manganese, which in rape seedlings in some concentrations stimulated and in some inhibited the glucobrassicin formation[19], in hypocotyl segments stimulated uptake of $^{35}SO_4{}^{2-}$ and in corresponding degree also the formation of glucobrassicin and especially of neoglucobrassicin. The inhibiting action of manganese on the incorporation of sulphate-sulphur into organic compounds therefore could not be confirmed.

Zusammenfassung. Die Biosynthese von Glucobrassicin und Neoglucobrassicin, insbesondere der Einfluss von Alloxan, Natrium- und Kaliummolybdenat auf die Inkorporation von Schwefel aus Sulfat, wurde untersucht.

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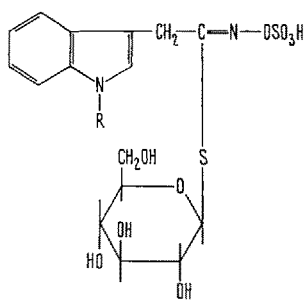

Glucobrassicin R=H
Neoglucobrassicin R=OCH3

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