were washed free of any adhering radioactivity and macerated in a Waring blender. The homogenate was allowed to stand for 1 h to allow enzymatic hydrolysis of the glucosinolate to take place. A few drops of concentrated hydrochloric acid were then added and the mixture steam-distilled, the distillate being collected in a large excess of aqueous ethanolic ammonia. The distillate was left at room temperature overnight to complete conversion of the isothiocyanate to benzylthiourea (BTU), and then evaporated to dryness. The residue was recrystallized to constant activity from aqueous ethanol. Activities were measured in a liquid scintillation spectrometer.

The results of the feeding experiments (Table I) show that both sodium phenylacetothiohydroxamate and sodium thioglucose appear to be highly efficient precursors,

Table I. Incorporation of sulphur containing compounds into benzylthiourea via the hydrolysis of non-isolated benzyl glucosinolate

Compound administered	Specific activity $\mu c/mM$	BTU specific activity $\mu c/mM$	% incorporation[a]	Dilution ratio[b]
Phenylacetothiohydroxamate-1-^{14}C	9.3	1.53	4.0	6.1
Phenylacetothiohydroxamate-^{35}S	18.8	6.21	4.8	3.0
Sodium thioglucoside-^{35}S	19.1	9.05	12.9	2.1

[a] % incorporation = (activity in BTU/activity absorbed by plant) × 100. [b] Dilution ratio = (specific activity of compound administered/specific activity of BTU).

Table II. Incorporation of phenylacetothiohydroxamate into benzylthiourea via the hydrolysis of crystalline benzyl glucosinolate

Phenylacetothiohydroxamate-^{35}S, specific activity $\mu c/mM$	Benzylthiourea, specific activity $\mu c/mM$	% incorporation	Dilution ratio
23.2	1.09	0.5	21.3

a conclusion that is incompatible with a single mechanism. The apparent incorporation of the phenylacetothiohydroxamate-^{35}S into benzyl glucosinolate was unexpected, and our first supposition was that the thiohydroxamic acid had decomposed to simpler compounds which were then incorporated by the pathway WETTER had demonstrated for sinigrin[5]. However, the carbon-14 labelled thiohydroxamate was also incorporated. Another possibility was that the phenylacetothiohydroxamic acid underwent a Lössen rearrangement in the plant, producing benzyl isothiocyanate which was still present at the time of extraction (or that this decomposition occurred during work-up). This possibility was investigated by feeding the plants with phenylacetothiohydroxamate-^{35}S and isolating the glucoside by the procedure of SCHULTZ and GMELIN[7]. The crystalline benzyl glucosinolate was hydrolysed by incubation with myrosinase to separate the 2 sulphur atoms. The isothiocyanate was isolated by steam distillation as described above. The results are shown in Table II. The low incorporation and high dilution obtained in this experiment strongly indicate that phenylacetothiohydroxamate is not a direct precursor of benzyl glucosinolate.

We therefore conclude that the thioglucoside moiety of benzyl glucosinolate is derived directly from thioglucose. This compound, which is not a common constituent of plants, probably arises by transfer of sulphur from one of the sulphur-containing amino acids to glucose[8].

Zusammenfassung. Phenylacetothiohydroxamat und Thioglukose wurden als mögliche biosynthetische Quellen des thioglukosidischen Schwefelatoms in Benzylglukosinolat (Glucotropaeolin, dem Senfölglukosid der Kapuzinerkresse (*Tropaeolum majus* L.) untersucht. Die experimentellen Ergebnisse deuten darauf hin, dass Thioglukose eine direkte Vorstufe ist, die mit einer von Phenylalanin abgeleiteten Stickstoffverbindung reagiert.

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Phosphorylation and Uphill Intestinal Transport of Thiamine, in vitro

The hypothesis that thiamine must be phosphorylated by the jejunal epithelial cells for its intestinal absorption, as we suggested in previous papers[1,2], has now been checked by some experiments in vitro, using ^{14}C-labelled thiamine and aspecific metabolic inhibitors or thiamine structural analogues, which specifically affect thiamine phosphorylation. The results we obtained by the everted intestinal sac technique are shortly reported here.

Everted sacs (8 cm long) from the upper small intestine of rats (Wistar strain, 100–150 g body weight) were prepared and incubated for 1 h in Krebs-Henseleit solution[3], as we previously described[1]. The initial concentration of thiamine(thiazole-2-^{14}C) hydrochloride (Radiochemical Centre, Amersham, England; specific activity 0.1 mc/1.26 mg)[4] at both sides of the sacs was 0.2 nM/ml and the

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Thiamine phosphorylation and net transport during intestinal uphill transport of thiamine-^{14}C by everted jejunal sacs of rats[a]. Averages ± S.E.

Concentration of inhibitors	No. of sacs	Thiamine phosphates in the wall		Net transport[b]	
		(nM/500 mg wet tissue)	Inhibition%[c]	(nM/500 mg wet tissue/h)	Inhibition%[c]
None (controls)	20	0.479 ± 0.029	0	0.265 ± 0.020	0
2,4-Dinitrophenol, $10^{-4}M$	12	0.104 ± 0.009	78.3	0.003 ± 0.001	98.9
Na fluoride, $10^{-2}M$	10	0.162 ± 0.024	66.2	0.058 ± 0.009	78.1
Oxythiamine, $2 \cdot 10^{-6}M$	13	0.478 ± 0.031	0.2	0.237 ± 0.016	10.6
Oxythiamine, $2 \cdot 10^{-5}M$	10	0.385 ± 0.044	19.8	0.235 ± 0.017	11.3
Pyrithiamine, $10^{-6}M$	11	0.148 ± 0.010	69.1	0.035 ± 0.007	86.8
2′-Ethylthiamine, $10^{-6}M$	12	0.149 ± 0.014	68.9	0.053 ± 0.017	80.0
2′-Butylthiamine, $10^{-6}M$	16	0.259 ± 0.015	45.9	0.093 ± 0.010	64.9

[a] The initial concentration of thiamine-^{14}C at both sides of the sacs was $0.2 \cdot 10^{-6}M$. [b] Net transport = the increase of thiamine (nM) in the serosal fluid after 1 h of incubation, expressed on a 500 mg of wet tissue basis. [c] 20% or less inhibitions are statistically not significant.

initial serosal volume was 1.2 ml. At the end of the incubation period, 0.5 ml of serosal fluid, diluted by washing with 50% ethanol to 5 ml, was placed on aluminium planchet and gently evaporated under an IR-lamp. The sac was then homogenized, in the cold, in 0.5N HCl with a Potter-Elvehjem glass homogenizer. The homogenate was centrifuged at 25,000 g in the multi-speed attachment of a refrigerated International Centrifuge, mod. PR-2. The supernatant, deproteinized with trichloroacetic acid (5% final concentration) and again centrifuged, was utilized for the chromatographic separation of thiamine phosphates following the slightly modified method of SHARMA and QUASTEL[5]. 0.5 ml of percolate was placed on a planchet and evaporated as above.

The radioactivity was determined with a Geiger-Müller gas-flow counter, using a micromil window (Nuclear, Chicago). The counting efficiency was 14–15%; the error of the measurements was less than 5%. The counts/min of each sample, corrected for the background, were converted into nM of vitamins divided by the counts/min of a labelled thiamine standard.

The metabolic inhibitors and thiamine analogues were initially added at both sides of the sacs in the concentration indicated in the Table, where all the results are summarized.

As can be seen, thiamine was phosphorylated by the intestinal mucosa during uphill transport in vitro. The aspecific metabolic inhibitors (2,4-dinitrophenol and Na fluoride) greatly reduced both net transport and phosphorylation of thiamine, the former being more affected than the latter. On the other hand, the effect of thiamine structural analogues (oxythiamine, pyrithiamine, 2′-ethylthiamine and 2′-butylthiamine) on transport was related to their action on thiamine phosphorylation: those analogues which depressed thiamine phosphorylation also inhibited thiamine uphill transport, the action on the transport being approximately proportional to that on phosphorylation.

In fact, oxythiamine, which does not affect thiamine phosphorylation by intestinal mucosa[6] and does not inhibit thiaminokinase purified from liver[7], affected neither thiamine phosphate content nor thiamine transport in our experiments. On the contrary, pyrithiamine, a strong inhibitor of thiamine phosphorylation by intestinal tissue[6] as well as of purified thiaminokinase[7], and 2′-ethylthiamine[8] and 2′-butylthiamine[8], both inhibitors of thiaminokinase[7], all at the same molecular concentration, lowered thiamine phosphates in the tissue, greatly reducing meanwhile thiamine uphill transport. It is noteworthy that the lack of action of oxythiamine was obtained with concentrations which were 2 and 20 times greater than those effective for other analogues.

Since none of the thiamine structural analogues had any effect on the glucose uphill transport in everted intestinal sacs, as we found in some separate experiments, their action on thiamine transport must be considered to be specific.

In conclusion, the results here reported indicate that thiamine was phosphorylated during uphill transport by everted intestinal sacs and that the inhibition of phosphorylation, obtained with metabolic inhibitors or with thiamine structural analogues, always reduced thiamine uphill transport. This is good evidence that thiamine phosphorylation is a basic mechanism for thiamine intestinal transport. However, the fact that the net transport was always more inhibited than thiamine phosphorylation (see Table) suggests that thiamine phosphorylation may not be the sole mechanism involved. Some experiments now in progress are devoted to investigating this point[9].

Riassunto. L'impiego di tiamina-^{14}C, con la tecnica dei sacchetti rovesciati di intestino di ratto, ha permesso di dimostrare che, durante il trasporto contro gradiente in vitro, la tiamina viene fosforilata dal tessuto intestinale. Gli inibitori metabolici e gli analoghi strutturali della tiamina che ne inibiscono la fosforilazione del pari ne riducono il trasporto netto. Ciò dimostra che il trasporto intestinale della tiamina è strettamente connesso con la sua fosforilazione.

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