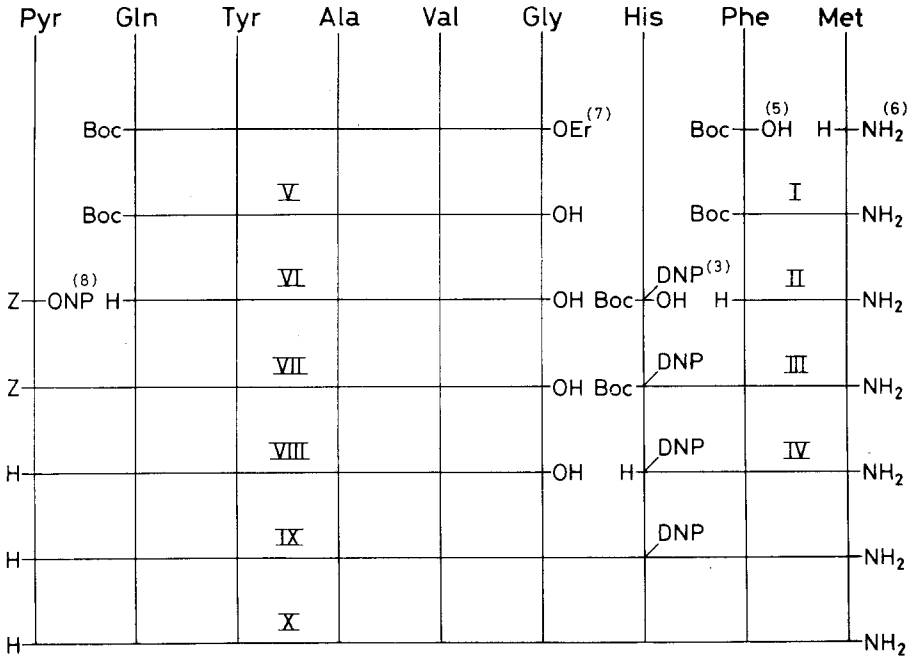

Synthesis of Litorin.

mobilities, the same degradative pattern and the same biological properties[4] of natural litorin[5–8].

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Riassunto. Viene riportata la sintesi della piroglutamil-glutaminil-triptofil-alanil-valil-glicil-istidil-fenilalanil-metioninamide, un peptide identico per proprietà fisiche, chimiche e biologiche alla litorina.

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H_2S as Sulfur Source in *Lemna minor* L.: II. Direct Incorporation into Cysteine and Inhibition of Sulfate Assimilation

Sulfate is the normal sulfur source of plants[1], but they are able to use other compounds to provide at least part of their sulfur requirements[2–4]. For Lemnaceae H_2S is of special interest, because they often grow on ponds where sulfide can be detected[5]. In these habitats, where H_2S is available to the plants together with SO_4^{2-}, an interesting question is how much the uptake and the assimilation of the oxidized compound is inhibited by the reduced one.

In a previous report we showed that H_2S inhibits the uptake of sulfate[6]. Here we shall present evidence for a direct incorporation of H_2S into cysteine. A simultaneous inhibition of the sulfate assimilation appears to be very probable.

Table I shows the result of an isotope-competition experiment: *Lemna minor* L. (strain-number 6580 of the collection of LANDOLT[7] were cultivated with radioactive $^{35}SO_4^{2-}$ in the nutrient solution and atmospheric air containing 0 or 6 ppm H_2S. After 10 and 15 days of cultivation, the specific activities of sulfate in the nutrient solution and in the plant material and of cysteine were determined. Cysteine was taken, because, from the quantitative standpoint, the most important pathway in the assimilation of sulfate is via cysteine[1].

The specific activity of sulfate and cysteine in the organisms cultivated with air is identical to the specific activity of sulfate in the nutrient solution.

In plants cultivated with air containing H_2S, the specific activity of sulfate in the plant material is only 1.25% that of the sulfate of the nutrient solution; H_2S provides the rest. The specific activity of cysteine is 50 times lower than that of sulfate present in the plant material, suggesting that 6 ppm H_2S almost completely blocks the assimilation of sulfate. This effect could be based on the inhibition of the enzymes of sulfate assimila-

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Table I. Specific activities of sulfate and cysteine after labelling *Lemna minor* with $^{35}SO_4^{2-}$ in air containing 0 or 6 ppm H_2S

Composition of air[a]	Duration of cultivation (days)[b]	Specific growth rate (μm)[c]	SO_4^{2-} of nutrient solution (μCi/μmole)[d]	SO_4^{2-} of plant material (μCi/μmole)[e]	Cysteine (μCi/μmole)[f]
20 l/h atmospheric air + 0 ppm H_2S	10	0.41	4.2	4.4	3.5
20 l/h atmospheric air + 6 ppm H_2S	10	0.35	4.1	0.041	0.0012
20 l/h atmospheric air + 0 ppm H_2S	15	0.39	4.2	3.9	4.3
20 l/h atmospheric air + 6 ppm H_2S	15	0.33	4.0	0.05	0.001

[a] H_2S-concentration produced by diffusion of H_2S through rubber tube into H_2S-free air and measured with a Jonoflux-detector (Hartmann und Braun, Frankfurt, BRD). [b] Cultivation in 150 ml Erlenmeyer flasks at 25 °C, 5000 lux (Philips TL 33) on 30 ml nutrient solution[20], pH 5.0 with a sulfate concentration of 4×10^{-4} *M*. [c] Growth was measured by counting the total number of fronds from daily photographs and the specific growth rate μm calculated according to

$$\mu = \frac{ln \text{ number of fronds } t_2 - ln \text{ number of fronds } t_1}{t_2 - t_1}$$

(t = time in days). [d, e] SO_4^{2-} determined after JOHNSON and NISHITA[21], radioactivity determined with a Liquimat 220 (Picker Nuclear). [f] Cysteine determined as adduct of N-ethylmaleimide-^{14}C after thinlayer chromatography in the solvent described by ELLIS[22].

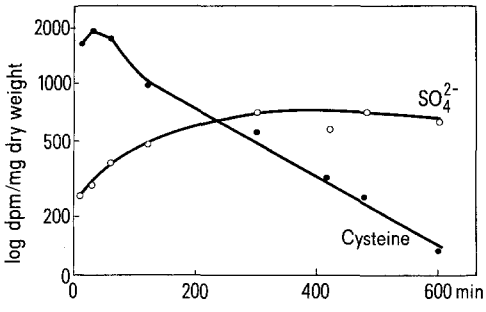
Table II. Formation of cysteine by extract[a] of *Lemna minor*

Reaction components	Cysteine formed in moles/min/mg protein
1. DL-Serine + pyridoxal-5-phosphate + $Na_2{}^{35}S$ + extract	2.1×10^{-10}[b]
2. *O*-acetylserine + Na_2S + extract	0.46×10^{-6}[c]

[a] Extraction according to ELLIS[2], protein determination according to LOWRY et al.[25]. [b] Assay according to SCHLOSSMANN[8], cysteine determined as adduct of N-ethylmaleimide after thinlayer chromatography in the solvent described by ELLIS[22]. [c] Assay according to BECKER et al.[9].

tion and a direct assimilation of H_2S, catalyzed by cysteine synthase (E.C. 4.2.1.22), an enzyme first found in yeast[8], or *o*-acetylserine sulfhydrylase, first found in *E. coli*[9]. Corresponding enzyme-activities were found in *Lemna minor*, using the methods of SCHLOSSMANN et al.[8] and BECKER et al.[9]. Table II shows that in vitro *o*-acetylserinesulfhydrylase has a much higher activity than cysteine synthase.

Both enzymes provide the possibility that *Lemna minor* assimilates H_2S directly. Experiments in vivo gave further evidence for a direct incorporation of H_2S. In a steady state experiment, *Lemna* plants cultured with



Radioactivity in cysteine and SO_4^{2-} in steady state experiment after pulse-labelling *Lemna minor* with radioactive $H_2{}^{35}S$ (specific activity 75 μCi/μmole) for 5 min. The organisms were cultivated with 6 ppm H_2S in a culture chamber which corresponds to the one described by ERISMANN and BRUNOLD[23], conditions as in Table I, except nutrient solution, in which SO_4^{2-} was replaced by Cl^-. The nutrient solution was constantly renewed. Radioactivity of cysteine as adduct of N-ethylmalinide and of sulfate was determined after thinlayer chromatography[22,24].

6 ppm H_2S were pulse-labelled with radioactive $H_2{}^{35}S$ for 5 min. Following the pulse, incorporation of ^{35}S into sulfate and cysteine extracted from the plant material shows the time course represented in the Figure. In cysteine, the radioactivity reaches a maximum 35 min after the radioactive pulse and then drops exponentially. The radioactivity of sulfate reaches its maximum after about 5 h and remains high during the following 5 h. This result suggests that $^{35}SO_4^{2-}$, formed by the oxidation of $H_2{}^{35}S$, is not a precursor of cysteine and is in agreement with the hypothesis of a direct incorporation of the H_2S fed.

In another type of experiment, *Lemna* plants were labelled with $^{35}SO_4^{2-}$ or $H_2{}^{35}S$ in the light or in the dark and examined for radioactive cysteine (Table III). When $^{35}SO_4^{2-}$ is the ^{35}S-source, the activity in the dark as percent of that found in the light is only 63% when labelling commences as darkness begins, or 43% after 60 min in the dark. This result is in agreement with the finding that the reduction of sulfate in green plants is enhanced by light[10–13]. The difference is not based on a difference in the rate of uptake of $^{35}SO_4^{2-}$ which is the same in the light and the dark.

With $H_2{}^{35}S$, the radioactivity of cysteine is the same in the light and in the dark. This fact can again be explained by a direct incorporation of the fed H_2S. If fed

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Table III. Formation of radioactive free cysteine from $^{35}SO_4^{2-}$ and $H_2{}^{35}S$ in light and darkness by *Lemna minor*[a]

Composition of air	^{35}S-source	Moles ^{35}S-cysteine/mg dry weight after 15 min with tracer		
		Light	Darkness before labelling (min) 0	60
Atmospheric air with 0 ppm H_2S	$^{35}SO_4^{2-}$	30×10^{-16}	19×10^{-16}	13×10^{-16}
Atmospheric air with 6 ppm H_2S	$H_2{}^{35}S$	25×10^{-12}	26×10^{-12}	24×10^{-12}

[a] Conditions as given in Table I. Before the experiment, the organisms were cultivated for 10 days with the indicated compositions of air.

H_2S were oxidized first to SO_4^{2-}, a similar difference between light and dark would be expected as was found when $^{35}SO_4^{2-}$ was the ^{35}S source.

Enzymes which can play a part in sulfate reduction in green plants were described only very recently[18,19]; future work with these enzymes might well discover that H_2S regulates SO_4-reduction in a similar way as NH_4 regulates NO_3-reduction[14–17].

Zusammenfassung. Bei *Lemna minor* L. hemmt H_2S in subtoxischen Konzentrationen die Assimilation von SO_4^{2-}. Es wird bei der Bildung von Cystein direkt eingebaut ohne vorangehende Oxidation zu SO_4^{2-} mit anschliessender Reduktion.

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Aminoacid Composition and Sequence of Litorin, a Bombesin-Like Nonapeptide from the Skin of the Australian Leptodactylid Frog *Litoria aurea*[1]

Methanol extracts of the skin of the Australian leptodactylid frog *Litoria* (*Hyla*) *aurea* contain a polypeptide, litorin, which possesses a bombesin-like activity. Litorin has been isolated in a pure form and recognized as a nonapeptide with the following sequence:

Pyr-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2

It may be seen that the C-terminal octapeptide of litorin is the same as that of ranatensin[2] and differs from that of bombesin or alytesin[3,4] in that, at position 2 from the C-terminus, a phenylalanine residue has been substituted for the leucine residue.

Isolation procedure. The dried skins of 230 specimens of *L. aurea* collected near Melbourne, Victoria, Australia, in November and December 1973, and weighing a total of 230 g, were subjected to 2 successive extractions with 20 parts (w/v) of 80% methanol, each extraction lasting 1 week. The extracts were mixed and filtered.

An aliquot of extract corresponding to 180 g of dried skins was evaporated to dryness, the residue washed with petroleum ether and then taken up in water plus 99% ethanol to give a final ethanol concentration of 95%. After standing, the limpid supernatant was passed through 4 columns of alkaline alumina, each of 170 g, which were then eluted with ethanol-water mixtures of decreasing concentrations of ethanol, each of 200 ml.

Two peaks of bombesin-like activity appeared in the ethanol eluates, one in the second 95% eluate, the other in the second 90% ethanol eluate. The study of the peptide responsible for the first peak is in progress; litorin was responsible for the second peak of activity.

The purification of the second 90% ethanol eluate, which was contaminated by 5-hydroxytryptamine and by smaller amounts of histamine, was carried out by using gel filtration on Sephadex G-10. Preparative paper electrophoresis was used as a final purification step.

On high voltage electrophoresis on paper, the active spot had the mobility of a positively charged peptide in neutral and acidic medium ($E_{5\cdot8} = 0.25$ His, $E_{1\cdot2} = 0.35$ Glu). The spot was negative to ninhydrin, denoting the absence of lysine and of a free N-terminal amino group, whereas it was positive to the Pauly reagent for histidine and to the reagents for tryptophan (*p*-dimethylaminobenzaldehyd and NNCD[5] reagents).

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