of the rabbit. From these observations we may conclude that the glucoside can be decomposed in the animal organism without the enzyme present in plants.

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The Enzymic Formation of Thiocyanate (SCN^-) from a Precursor(s) in *Brassica* Species

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The chemical nature of the goitrogenic "*Brassica* factors" is still unclear to a great extent. This is partly due to the fact that several active factors may be involved, which are moreover formed only through enzymic reactions when plants are crushed. This has long been known in regard to the formation of the strongly goitrogenic thiooxazolidones occurring in the crushed and moistened seeds of many crucifers [1]. Recently Virtanen *et al.*[2], and Kreula and Kiesvaara [3] found the formation of vinyl-thiooxazolidone also in cabbage, kale, rape, and other fodder plants belonging to the *Cruciferae* family.

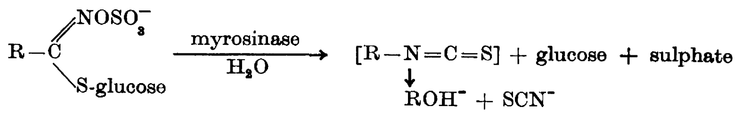
The same authors [4] have also demonstrated the transfer of this substance to milk in very small amounts (about 0.05 % of the amount fed). These quantities are so small that they cannot be expected to have any goitrogenic effect in the milk. Kreula and Kiesvaara [4] have given a detailed report on the methods used in these investigations. Somewhat later Altamura *et al.*[5] independently also demonstrated the formation of vinyl-thiooxazolidone in cabbage.

The goitrogenic effect of thiooxazolidones is due to the fact that they inhibit the synthesis of thyroid hormones. This effect cannot be overcome by high doses of iodide.

Another type of goitrogenic substances belonging to the *Brassica* factors has a primary influence on the uptake of iodide by the thyroid gland. This influence can be prevented by increasing the amount of the iodide dose. Many pieces of information given in the literature [6,7] indicate that salts of thiocyanic acid (SCN^-) represent this type in crucifers. Jirousek [8], who has investigated and reviewed the thiocyanate metabolism, found that in the animal organism SCN^- is formed endogenously from cyanides, nitriles, and sulphur-containing compounds, and that both the SCN^- brought exogenously into the organism and that formed endogenously in it have to be taken into consideration as goitrogenic factors. Michajlovskij and Langer [9,10] have performed systematic determinations on the SCN^- content in different vegetables. They found a particularly high SCN^- content in the press juice of different cabbage species (up to 50 mg %). They used the term "präformiertes Rhodanid" for the SCN^- present in food stuffs, obviously in contrast to the SCN^- formed endogenously.

Our investigations concerning the problem whether it is possible to make milk goitrogenic by feeding cows with plants, especially those belonging to the *Cruciferae*, have led to some new findings regarding the *Brassica* factors. These findings are briefly reported in the present paper.

An account was recently given about the formation of organic thiocyanates in some crucifers [11]. Benzyl thiocyanate was found to be formed enzymatically from glucotropaeolin in *Lepidium ruderale* and allyl thiocyanate from sinigrin in *Thlaspi arvense.* It was found in this laboratory that after the injection of benzyl thiocyanate into rats, the SCN^- content has risen considerably in blood serum and in different organs. This finding led us to look for thiocyanate esters also in *Brassica oleracea* species. For the present we have, however, no indications for the formation of such esters in fresh cabbage species. On the other hand, the result of these investigations was the finding that free SCN^- is formed from glucosidic precursors, present in cabbage. The thiocyanate found by Michajlovskij and Langer in the press juice of cabbage is thus not "preformed SCN^-"



but is formed enzymatically from a glucosidic precursor(s) in cabbage. This fact was established when enzymes were destroyed in cabbage leaves by placing them intact in boiling methanol. The solution obtained was free from SCN^-. After addition of myrosinase solution to the purified extract SCN^- was formed, as determined by colour reactions for SCN^- and by paper chromatography.

The determination of SCN^- formed enzymatically in fresh plants was performed in the following way. 10 g of whole leaves of *Brassica oleracea* species were twice extracted by boiling for 15 min in 50 ml portions of methanol. The methanolic solutions were decanted. The plant material was thoroughly ground in a mortar, and was once more extracted by boiling in 50 ml of 70 % methanol. The combined extracts were evaporated *in vacuo*. The residue was taken up in water, and the solution was treated with about 0.6 ml of 20 % lead acetate solution. Filtration and washing of the filter residue with water. Excess lead ions in the filtrate were precipitated by H_2S. The filtrate was concentrated *in vacuo* to about 20 ml and brought to 25 ml (volumetric flask). 10 ml of this solution, 1 ml of myrosinase solution *, and 1 ml of phosphate buffer at pH 7 were incubated for 2 h at 37°. The solution was then brought to 100 ml. A control without addition of myrosinase solution was treated in the same way, and was used as a blank. 5 ml samples were mixed with 1 ml of Fe-reagent, measured at 500 mμ with a Klett-Summerson colorimeter, and the values compared with a standard SCN^- curve, essentially as in the method of Barker [13].

Table 1 shows the amounts of SCN^- formed in different cabbage species.

As is apparent from the list, varying amounts of SCN^- are formed in different cabbage species. The thiocyanate content of the press juices found by Langer and Michajlovskij corresponds to the amounts found by us.

* prepared according to Neuberg and Wagner [12]. SCN^- originally present in the myrosinase solution, was removed by shaking the solution for 30 min with Dowex 2 × 4 in chloride form.

Table 1. SCN^- content in cabbage species after myrosinase treatment.

Brassica species	SCN^- formation per 100 g fresh plants
Brassica oleracea * var. *sabauda* ssp. *Ulmer*	27—31 mg
Brassica oleracea * var. *gemmifera*	10 mg
Brassica oleracea * var. *capitata*	4 mg
Brassica oleracea * var. *cretica*	4 mg
Brassica napus var. *rapifera* •	8.8 mg
Brassica napus * var. spring rape	2.5 mg
Brassica rapa * var. winter turnip rape	1.7 mg

* leaves
• root

No formation of SCN^- was found in the crushed and moistened seeds of different cabbage species.

Attempts to isolate the glucosidic precursor from cabbage have been unsuccessful so far. The following findings can, however, be presented.

1. The precursor(s) of SCN^- decomposes with myrosinase so that SCN^- is liberated.
2. On spraying with $AgNO_3$ solution and heating, the precursor of SCN^- gives the same reaction on the paper chromatogram as mustard oil glucosides (gray-brown spot[14]).
3. On spraying with $FeCl_3$ solution, the precursor of SCN^- gives a brownish red spot after a short heating to 100°.
4. The position of the precursor on the paper chromatogram could be established furthermore either by spraying the paper first with myrosinase solution, and after standing for 1 h with $FeCl_3$ solution or by eluting the zones by the usual colour reactions for SCN^- after treatments of the eluates by myrosinase.

5. In a butanol-acetic acid-water system, the R_F value [15] of the SCN^- precursor was 0.76, in a pyridine-amylalcohol-water system it was 1.0.

Although the information about the chemical nature of the precursor is still scanty, we think it reasonable to publish a preliminary communication about our results at this stage. In our opinion the finding of the enzymatic formation of SCN^- is rather important, since it may help to shed light on many discrepancies about the *Brassica* factors.

If the SCN^- precursor is a mustard oil glucoside, as we suppose on the basis of the present indications, the formation of SCN^- according to the following reaction scheme seems probable, (see p. 508).

This secondary decomposition of an *iso*thiocyanate is no unknown reaction. Already in 1879 Will and Laubenheimer[16] have observed that *p*-hydroxybenzyl mustard oil (the *iso*thiocyanate formed from the glucoside sinalbin, present in white mustard) is split almost quantitatively into SCN^- by the action of alkali.

We have, however, found that considerable amounts of SCN^- are formed from glucosinalbin during the enzymatic cleavage even at pH 7. The initially present, pungent tasting *p*-hydroxybenzyl isothiocyanate decomposes gradually, loosing its irritating properties, into SCN^- and probably *p*-hydroxybenzyl alcohol. This secondary reaction seems to proceed at pH 7 at a slower rate than is the case with the SCN^- precursor of cabbage. Treatment of glucosinalbin with alkali, however, affords complete and fast fission, and permits a simple assay for the glucosinalbin content in the seeds of *Sinapis alba* by colorimetric SCN^- determination.

As long as the SCN^- precursor has not yet been isolated from *Brassica oleracea* species, the plants containing glucosinalbin (*Sinapis* species, *Bunias* species, *Lepidium campestre etc.*) may be used as suitable models for studies on the goitrogenic effect of the SCN^- precursor. Wagner-Jauregg and Koch [17] have for example observed a slight goitrogenic effect by application of myrosinase treated sinalbin solution to rabbits (sinalbin itself was without effect) but have not recognized or regarded SCN^- as the evidently responsible goitrogenic factor in this experiment.

Several thioglucosides were found in the limited number of cabbage species used so far for the attempted isolation of the SCN^- precursor: glucoraphanin, sinigrin, and gluconapin in the fresh parts of *Brassica oleracea* var. *gemmifera;* glucoiberin and sinigrin in the fresh parts of marrow stem kale ("Chou Moellier"); glucoiberin and glucoraphanin in *Brassica oleracea* var. *cretica.* They were detected [14] directly by paper chromatography and comparison with authentic thioglucosides, or indirectly as thioureas derived from the enzymatically formed *iso*thiocyanates by the method of Kjær and Rubinstein [18] in different solvent systems.

From 500 g fresh parts of *Brassica oleracea* var. *gemmifera* 15 mg of analytically pure sulphoraphan-phenylthiourea were isolated (m. p. 145°C) *.

The presence of glucoiberin and glucoraphanin and their *iso*thiocyanates, respectively, in cabbage species becomes of special interest since Bachelard and Trikojus [20] have recently found that cheirolin (3-methylsulphonylpropyl *iso*thiocyanate) has a significant thyreostatic effect in animals. Iberin (3-methylsulphinylpropyl *iso*thiocyanate) and sulphoraphan (4-methylsulphinylbutyl *iso*thiocyanate) belong to the same isothiocyanate type as cheirolin, and may thus be considered as potential members in the group of "Brassica factors".

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Isolation of an Iron-Containing Red Protein from Human Milk

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The presence of a red protein in bovine milk has been observed by several authors [1-4]. Sörensen and Sörensen [1] as well as Polis and Shmukler [3] performed a partial purification of this protein and recently a procedure for its isolation was described by Grooves [4]. The protein isolated by Grooves contained iron and was in certain respects similar to the iron-binding protein in blood plasma (transferrin [5] or β_1-metal-combining protein [6]). According to Schäfer [7] *human* milk assumes a red color after addition of small amounts of iron, probably due to the formation of an iron-protein complex. It has also been observed in this laboratory that crude preparations of human milk whey proteins are often salmon coloured[8]. This colour becomes more pronounced after addition of iron to the milk. A partial purification of the red component has been achieved by chromatography on calcium phosphate [9]. A method for isolation of the red protein has now been worked out and will be presented below.

Four litres of fresh human milk were defatted by centrifugation. After addition of 10 mg of Fe^{2+} (in the form of ferrous ammonium sulphate) to the milk, giving it a distinct reddish tint, solid ammonium sulphate was added to 45 % saturation and pH adjusted to 8.0 by careful addition of concentrated ammonia. When the milk was warmed to 37° for 30 min a flocculent precipitate was formed, which was removed by filtration through a fluted paper (Whatman No. 12). The rate of filtration was most often slow and the filtration had to proceed overnight at +4°C. The precipitate was discarded and the red-brown filtrate was acidified to pH 4.0 with 1 M sulfuric acid. The precipitate formed was quickly removed by gravity filtration or better by centrifugation and the pH of the filtrate (supernatant) immediately readjusted to 8.0. Ammonium sulphate was added to 80 % saturation and the red precipitate collected by filtration and dissolved in a minimal amount of distilled water. The dark red solution was dialyzed against distilled water followed by dialysis against 0.02 M sodium phosphate buffer, pH 8.0. The solution was then chromatographed on DEAE-cellulose [10] previously equilibrated with the same buffer. Under the conditions described the red protein was not sorbed on the ion-exchanger but appeared in the effluent without significant retardation. The red fraction was further chromatographed on calcium phosphate as described earlier [9], using phosphate buffers of pH 8.0 instead of 6.9. The red fraction eluted with 0.5 M phosphate buffer was dialyzed against distilled water and finally

Fig. 1. Electrophoretic pattern of the purified red protein. Glycine-NaOH buffer, pH 9.4, ionic strength 0.1. Ascending boundaries to the left, descending boundaries to the right. Duration 350 min.