TABLE I.
Thyroid Hyperplasia of Rats after 1, 2, or 3 Months of Feeding with Soy Bean-Millet Diet Supplemented by Dried Thymus, Beef, Ox Liver, or Cod Spawn Respectively

Diet No.	Avg size of thyroid in % of norm. for groups 5 rats for 30, 60, and 90 days respectively			Cellular hyperplasia of thyroid, estimated for each individual group as an entirety		
1. Dried thymus	123	130	126	+	++	++
2. Thymus, extr. by alcohol	120	124	136	++	++	++
3. Water soluble fract. of thymus alc. extr.	143	108	130	+	0	+
4. Water insolub. fract. of thymus alc. extr.	99	140	138	0	+	+
5. Dried beef	117	144	124	0	0	0
6. Dried liver	137	116	113	0	0	+
7. Alcohol extr. of ox liver	111	153	123	0	+	+
8. Dried cod spawn	115	106	108	0	0	0

to the increase in the size of the thyroid, whereas the histological changes, *i.e.* the cellular hyperplasia and especially the reduction of colloid substance became particularly pronounced.

Summary. Moderate cellular hyperplasia of the thyroid was produced in rats on a soy-bean-millet diet or a usual standard diet by adding dried thymus from calves.

The goitrogenic fraction was insoluble in water and ethyl alcohol; no goitrogenic effect was observed after feeding with dried ox liver, dried beef or dried cod spawn.

16177

Isolation and Properties of Raphanin, an Antibacterial Substance from Radish Seed.

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Antibacterial substances are not rare in various higher plant species.[1] In the course of a systematic investigation of aqueous extracts of plants belonging to the family of Cruciferae for the presence of antibiotic principles we found[2] that extracts of the seeds of radish (*Raphanus sativus*), when tested by the cylinder plate method (Heatley[3]), gave a wide zone of inhibition on plates seeded either with *Staph. aureus* or with *B. coli.* The substance responsible for the action could be purified and finally isolated as a homomolecular liquid.

Experimental. The aqueous extract of the seeds is very effective in preventing the growth of bacteria. The cell-free extract prepared by the extraction of one part of finely ground seeds with 5 parts of water gave a 30 mm zone of inhibition on plates seeded with *Staph. aureus* or *B. coli.* Even extracts prepared in the ratio of 1:50 caused marked inhibition. The antibacterial principle is fairly resistant to heat; only a slight decrease in potency was observed after heating it to the boiling point for 30 min. The cell-free extract will retain full activity for weeks if stored in the ice box. However, all activity is lost within 24 hours if the extract is incubated with the crushed seeds at 37°C. Considerable inactivation occurs under such conditions even at 20°C; in fact, even at ice box temperature. On the other hand, heating the suspension to 60°C for 15 min. completely prevents inactivation.

1 Kavanagh, F., *Adv. Enzymology,* 1947, **7**, 461.
2 Ivánovics, G., and Horváth, S., *Nature,* 1947, **160**, 297.
3 Heatley, N. G., *Bioch. J.,* 1944, **38**, 61.

TABLE I.
Extraction of Antibiotic by Various Solvents from Aqueous Extract of Seeds.

Solvent	pH of extract	Diameter of zone of inhibition in mm: Dilution of organic solvent fraction 1:1	1:2	1:3	Dilution of aqueous residue 1:4	1:8	1:12
Amyl acetate	2	24	22	21	22	16	15
" "	3	24	22	20	20	17	15
" "	4	26	24	19	21	17	14
Butanol	3	27	26	26	16	14	8
Ethyl acetate	3	28	26	25	22	16	13
Petroleum ether	3	13	9	0	27	22	20

If intact or carefully peeled seeds are soaked in water for a few hours the diffusate is found to be devoid of activity, thus indicating that the active principle is firmly bound to the cells. Since the active substance obtained from crushed seeds passes a cellophane membrane with ease it is obvious that the seeds do not contain the active principle proper. This fact, together with the heat resistance of the active extract and with the failure to obtain active extracts from seeds boiled 15 min. before crushing them, led to the assumption that the seeds contain an enzyme capable of converting an inactive precursor into an antibiotic. The correctness of this assumption could be proven by the following experiment: Crushed seeds were extracted with 3 changes of 80% ethanol; the extract was concentrated and freed from alcohol *in vacuo* (extract A). Another portion of the crushed seeds was extracted with water and dialyzed in a cellophane bag against tap water (extract B). Both extracts were entirely devoid of any antibacterial activity. However, when the extracts were mixed, and the mixture allowed to stand for one hour, a potent solution resulted. No activity was obtained if extract B was heated to 70°C for 30 min. before adding it to extract A.

Crude extracts of the active principle were prepared as follows: 2000 g of finely ground seeds was stirred up with 3000 ml of tap water, and the mixture allowed to stand at room temperature for 30 to 40 min. The suspension was strained through cheesecloth, and the residue squeezed out in a hand press. The combined filtrates were adjusted to pH 5, and 14 to 15 ml of a 40% solution of basic lead acetate was added to each 100 ml of the juice. The precipitate was centrifuged off and discarded. The excess lead, which otherwise would have caused gradual inactivation, was removed by the addition of 7 to 8 ml of a 20% solution of $Na_2HPO_4 \cdot 12H_2O$. The supernatant gave only a very slight reaction with H_2S. It should be remarked here that the excess lead must not be removed with H_2S to which the active principle is sensitive. This procedure of purification caused a loss not exceeding 20%. The extract thus obtained will be referred to as the purified aqueous extract.

The active principle can be extracted with ease by various organic solvents at an acid pH. 40 ml of the purified aqueous extract was shaken with 20 ml of various organic solvents. The solvents were subsequently evaporated *in vacuo,* and the residue redissolved in 10 ml of water. Both the original aqueous phase and this latter solution were assayed by the cylinder plate method. The results are shown in Table I where the concentration of the redissolved organic solvent fraction is referred to as 1:1, while that of the original aqueous phase is taken as 1:4, in comparison to the other fraction.

The active principle is not absorbed on tricalcium phosphate or on kaolin. However, it is easily adsorbed on charcoal at pH 5 to 7. All attempts at elution between pH 2.2 and 11 failed.

In further experiments a butanol concentrate of the active principle served as a standard of reference. An amount producing a 19 to 21 mm wide zone of inhibition was chosen as an arbitrary unit of activity.

The pure active principle was isolated in

TABLE II
Elementary Composition of Raphanin and of Inactive Crystalline Substance.

Solvent used for extraction	Substance	% composition							
		C		H		N		S	
Butanol	Raphanin, Batch 3								
	First fraction	41.71		5.03					
		41.10	41.27*	5.41	5.34*				
		41.00		5.58					
	Middle "	41.23	41.26*	5.28	5.18*	8.41			
		41.30		5.08					
	End "	41.66		5.27					
Butyl acetate	Raphanin, Batch 5†	41.62	41.50*	5.61	5.50*	8.91		33.94	33.64*
		41.38		5.40				33.34	
	Inact. cryst. substance	34.64		5.48		6.89		45.42	45.36*
		34.71	34.65*	5.09	5.31*	6.70	6.73	45.31	
		34.60		5.35		6.60			
Theoretical values for	$C_{17}H_{26}N_3O_3S_5$	42.48		5.45		8.74		33.35	
	$C_{17}H_{26}N_3O_4S_5$	42.11		5.28		8.58		33.03	

* Average values
† Freshly redistilled by molecular distillation.

the following manner: 5330 ml of the purified aqueous extract containing 7370 units of the antibiotic was obtained from 2000 g of seeds. This solution was extracted with six 1000 ml portions of butyl acetate. The extracts were combined and assayed; total recovery was 5648 units. The solvent was removed by vacuum distillation. The resulting brown oily material was shaken with 200 ml of 0.07M phosphate buffer pH 7.2; the insoluble material was filtered off and discarded. The water-soluble fraction was extracted with 3 portions of chloroform, 30 ml each. The combined chloroform extracts contained 5500 units. The solution of the antibiotic in chloroform was shaken with 100 ml of phosphate buffer to remove the last traces of acidic impurities. The active principle, together with most of the pigment, remained in the chloroform layer. The solution was now passed through a column of Brockman's alumina which adsorbed a brick-red pigment. The percolate contained all the activity. After evaporation of the chloroform and of the last traces of moisture, a slightly yellow liquid of syrupy consistency was obtained. It had a sharp boiling point and could be distilled under reduced pressure without much decomposition. Boiling point: 135°C at 0.06 mm; 142.5°C at 0.09 mm, and 145°C at 0.1 mm Hg. Total yield, 7.5 g.

The distillate which consisted of the pure active principle was designated as raphanin. The homogeneity of the distillate could be established by redistillation at 135°C under a pressure of 0.06 mm Hg. Fractions were collected separately at intervals at the beginning, the middle and the end of the procedure and analyzed for C, H, N and S (Table II).

Raphanin is only moderately soluble in ether. The partition coefficient between butanol and water is favorable but butanol extracts contain a large amount of impurities which can be removed only with difficulty.

In an effort to reduce the amount of solvent required for extraction, the purified aqueous extract was concentrated *in vacuo* at 60°C to about one-half of its original volume. In one experiment 4500 ml of purified aqueous extract was reduced to 2000 ml. This operation resulted in the loss of about 1000 units. The concentrated liquid was extracted with butyl acetate; the solvent was evaporated *in vacuo* to a small volume. This concentrate was placed in the refrigerator, and after standing there overnight, a crop of crystals appeared. The crystals were collected, recrystallized first from boiling water, then from ethanol. Yield, 400 mg M. P. 192°C (decomp.). The elementary analysis is shown in Table II. This crystalline substance did not possess any antibacterial activity.

Some physical and chemical properties of raphanin. Freshly distilled raphanin has a

TABLE III.
Highest Dilutions of Raphanin Causing Inhibition of Bacterial Growth.

Organism	Medium	Partial inhibition × 1000	Complete inhibition × 1000
Staph. aureus	broth	1:50	1:28
'' ''	serum broth*	1:1	1:1
'' ''	casein hydr. broth†	1:50	1:28
B. coli	broth	1:10	1:5
'' ''	serum broth*	1:1	1:1
'' ''	synthetic‡	1:125	1:70
Salm. schottmülleri	broth	1:8	1:5
B. anthracis	''	1:8	1:4
Pseud. aeruginosa	''	1:16	1:8
'' ''	serum broth*	1:4	1:2
Salm. typhi	broth	1:16	1:8
'' ''	serum broth*	1:4	1:2

* Containing 50% ox serum.
† According to Iványovics, *Acta Med. Szeged*, 1944, **12**, fasc. 1.
‡ According to Sahyun, Beard, Schultz, Snow, and Cross, *J. Infect. Dis.*, 1936, **56**, 58.

TABLE IV.
Zones of Inhibition in mm with Various Concentrations of Raphanin.

Organism	Zone of inhibition in mm		
	1 mg/ml	2 mg/ml	3 mg/ml
Staph. aureus	18.5	22.5	24
B. anthracis (avirulent)	23	26.5	28
'' '' (virulent)	22	26.5	29
'' *subtilis* (strain Duthie)	20	26	29
'' *coli*	16	19.5	21
Salm. typhi—H	19	24.5	27
'' '' —O	24	26.5	28.5
'' *schottmülleri*	22	28.5	31
B. shiga-kruse	16	20	22
Pseud. aeruginosa	19	23	25
B. prodigiosus	12	15	19

slightly yellowish shade which will darken on storage at room temperature. It was attempted to obtain a colorless sample by molecular distillation at 60°C. The distillate had a very minimal yellowish tinge; it was impossible to secure an entirely colorless preparation. It is believed that the yellowish shade is due to decomposition products although darkening at room temperature is not associated with any noticeable loss in antibacterial activity.

The substance has a radish-like odor. It is quite soluble in water with a neutral reaction. Its solution in absolute ethanol is levorotatory; $[\alpha]_D^{20} = -141°$. Freshly prepared aqueous solutions give no coloration with ferric chloride or with nitroprusside. However, after treatment with dilute HCl and Zn dust, an intense purple shade was obtained with nitroprusside, indicating the presence of –SH groups. If the aqueous solution is heated with Ag or Pb salts, a heavy black precipitate, insoluble in HNO_3, is obtained, and the supernatant is found to have lost its antibacterial activity.

If raphanin is shaken with 0.1N $Ba(OH)_2$ at room temperature, the excess alkali neutralized with H_2SO_4, the $BaSO_4$ precipitate removed by filtration, and the filtrate, evaporated to a small volume, is placed in the refrigerator, crystals will precipitate on standing. This crystalline substance is inactive. M.P., after recrystallization from ethanol, is 192-93°C (decomp.). When these crystals were mixed with those obtained from the butyl acetate concentrate, no depression of the melting point was observed.

Antibacterial activity of raphanin. Raphanin was assayed on a number of bacterial species, using both the serial dilution method

TABLE V.
Residual Antibacterial Activity of Raphanin After Heating Its Solutions for 30 min. at Differenut pH (expressed in per cents of original activity).

Buffer	pH	Temperature 20	60	80	100°C
Phosphate	5.3	100	100	85	40
"	7.2	100	80	50	0
Glycine	8.0	90	75	50	0
"	10	50	0	0	
"	11	40	0	0	

and the cylinder plate technique. An aqueous solution of freshly distilled raphanin was sterilized by filtration through a fritted glass plate. Various amounts of raphanin were added to 4 ml of nutrient medium, and the volume was made up with dist. water to 5 ml. The tubes were inoculated with 0.1 ml of a dilute bacterium suspension containing about 50,000 organisms in one ml. They were incubated for 24 hours at 37°C. (Table III). In tests by the cylinder plate method 3 different dilutions containing 1, 2 and 3 mg of raphanin per ml were used (Table IV).

The activity of raphanin against *B. coli* is superior to that of crystalline penicillin G since a 1:1000 dilution of raphanin gave a zone of inhibition of 18 mm, whereas penicillin in the same dilution gave a zone of only 13 mm. Even a 1:5000 dilution of raphanin produced noticeable inhibition while penicillin in a strength of 1:3000 was without effect.

Raphanin is readily inactivated in the alkaline range while it is much more stable at a neutral or slightly acid solution. 25 mg of raphanin was dissolved in 5 ml of buffer solution; the solutions were incubated at various temperatures for 30 min, and the activity was tested by the cylinder-plate method (Table V).

As mentioned, raphanin is rapidly inactivated by H_2S. However, this activation is dependent on the pH of the medium. If H_2S is bubbled through a buffered solution of raphanin for 3 min, and the gas subsequently removed by aeration, complete inactivation occurs at pH 7.2, but only 50% inactivation at pH 5.

Toxicity of raphanin. Owing to an acute shortage of experimental animals, the toxicity of raphanin could be tested only in a small number of mice and guinea pigs. Mice weighing 16 to 22 g were injected i.v. with doses of raphanin ranging from 5 to 40 mg. Five mg caused only transitory weakness, excitement and ruffled fur, and the animals recovered promptly. Doses of 7 to 10 mg killed the animals within 5 to 10 min, while doses of 20 to 40 mg caused death in a few seconds. The results were very similar when raphanin was injected subcutaneously, except that the appearance of the symptoms was somewhat delayed. The symptoms after the injection of a lethal dose were weakness, restlessness, dyspnea and clonic convulsions. Animals injected with 10 to 20 mg scratched their noses furiously with their hind legs. At post-mortem examination no gross anatomical changes were found, except for a slight edema at the site of injection.

Three guinea pigs, weighing 450 to 470 g, were injected intracardially with 25, 50 and 125 mg. No ill effect was observed after 25 mg; 50 mg caused death after 30 min, while the injection of 125 mg was followed by signs of weakness and excitement, and the animal died in convulsions after a few minutes.

In tissue cultures of rabbit testis, a 20,000 dilution of raphanin completely prevented the growth of fibroblasts; there was a definite inhibition of growth at a dilution of 1:40,000, while a dilution of 1:80,000 did not cause any inhibition.

Raphanin is a potent inhibitor of the germination of various plant seeds, including those of radish itself. Seeds were placed on discs of filter paper soaked in dilute solutions of the antibiotic. The discs were kept in a moist chamber for 3 days at room temperature. Results are shown in Table VI.

TABLE VI.
The Germination of Seeds of Different Plants in the Presence of Raphanin.

Plant species	Conc. of raphanin	No. of seeds	No. of seeds germinated	Avg length of roots in mm
Raphanus sativus	0 (control)	25	23	21.5
" "	1:200	20	1	3.0
" "	1:1,000	25	24	14.0
" "	1:10,000	25	25	20.0
Brassica oleracea	0	25	20	17.2
" "	1:1,000	25	8	4.0
" "	1:10,000	25	20	11.0
Festuca pratensis	0	20	14	8.6
" "	1:1,000	20	1	4.0
" "	1:10,000	20	13	5.3
Sinapis alba	0	25	19	10.2
" "	1:1,000	25	0	0
" "	1:10,000	25	12	12.5
Hordeum distichon	0	20	20	16.0
" "	1:1,000	20	6	7.7
" "	1:10,000	20	20	12.8

Comments. The molecular weight of raphanin has not been estimated yet but on the basis of data of the elementary analysis the empirical formula could be either $C_{17}H_{26}O_3N_3S_5$ or $C_{17}H_{26}O_4N_3S_5$. The negative nitroprusside reaction indicates the absence of –SH groups and rules out the existence of an unsaturated lactone ring in the molecule. On the other hand, the appearance of –SH groups after reduction with nascent hydrogen is suggestive of a disulfide linkage.

The seeds of some of the Cruciferae contain thioglucosides which are hydrolyzed by concomitant enzymes into sugars and aglycons, the latter being mustard oils (isothiocyanic esters). This fact is of importance because it is asserted[4] that allyl isothiocyanate and related mustard oils possess an antibacterial action, a statement not borne out by other observations.[5] We were also unable to demonstrate any antibiotic effect in the seeds of *Sinapis alba*. Raphanin, as can be judged from its composition and chemical behavior, does not seem to belong in the group of mustard oils, although data so far obtained do not permit any definite conclusions as to its exact structure. It bears some resemblance to gliotoxin isolated from *Aspergillus fumigatus* and to allicin. Both of these latter antibiotics contain a high percentage of sulfur in a disulfide linkage; both are effective against Gram-positive as well as against Gram-negative organisms, and both are rather toxic. It is interesting to note that the intact cells of garlic do not contain allicin but only an inactive precursor which is transformed by a concomitant enzyme into active allicin.[6]

Summary. The preparation and purification of a new antibiotic, raphanin, from the seeds of the radish is described. The seeds contain an inactive precursor which is activated to a potent antibiotic by a concomitant enzyme.

The physical, chemical, antibiotic and toxic properties of raphanin are described. Raphanin, owing to its high toxicity, does not hold the promise of a useful therapeutic agent.

[4] Foter, M. J., and Golick, A. M., *Food Res.*, 1938, **3**, 609.

[5] Osborn, E. M., *Brit. J. Exp. Path.*, 1943, **24**, 227.

[6] Cavallito, C. J., Bailey, J. H., and Kircher, F. K., *J. Am. Chem. Soc.*, 1944, **66**, 1950.