ON THE OCCURRENCE OF GROWTH INHIBITING SUBSTANCES IN RYE

Dit proefschrift met stellingen van

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landbouwkundig ingenieur, geboren te Bussum, 20 april 1924, is goedgekeurd door de promotors Dr. C. den HARTOG, hoogleraar in de leer van de voeding en de voedselbereiding en Ir. S. IWEMA, hoogleraar in de leer van de veevoeding.

De Rector Magnificus van de Landbouwhogeschool,
F. HELLINGA

Wageningen, januari 1967

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ON THE OCCURRENCE OF GROWTH INHIBITING SUBSTANCES IN RYE

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWKUNDE
OP GEZAG VAN DE RECTOR MAGNIFICUS, IR. F. HELLINGA,
HOOGLERAAR IN DE CULTUURTECHNIEK,
TE VERDEDIGEN TEGEN DE BEDENKINGEN
VAN EEN COMMISSIE UIT DE SENAAT
VAN DE LANDBOUWHOGESCHOOL TE WAGENINGEN,
OP VRIJDAG 24 FEBRUARI 1967 TE 16.00 UUR

DOOR

G. W. WIERINGA

H. VEENMAN EN ZONEN N.V. – WAGENINGEN – 1967

Aan mijn Vader
aan de nagedachtenis van mijn Moeder

STELLINGEN

I

Rogge kan, ondanks het vóórkomen van 5-n-alkylresorcinolen, nauwelijks als schadelijk voor dieren worden aangemerkt. Het kan onder bepaalde omstandigheden zelfs aanbeveling verdienen 10-20% rogge in rantsoenen voor biggen op te nemen.

II

De algemeen verbreide mening, dat stomen de schadelijkheid van rogge doet afnemen, is niet juist.

Dit proefschrift
HUTCHINSON, J. B., T. MORAN and J. PACE, J. Sci. Fd. Agric. *15* (1964) 413–417

III

De mening van WENCKERT en medewerkers dat in tarwezemelen naast 5-n-alkylresorcinolen niet ook 5-n–alkenylresorcinolen voorkomen, is aan twijfel onderhevig.

Dit proefschrift
WENCKERT, C., E. M. LOESER, S. N. MAHAPATRA, F. SCHENKER and M. WILSON J. Org. Chem. *29* (1964) 435

IV

Het verdient geen aanbeveling gekleurd licht te gebruiken bij de zintuigelijke beoordeling van levensmiddelen.

WIERINGA, G. W., Voeding *25* (1964) 205–216

V

Het toevoegen van amylase preparaten aan in te kuilen gras kan leiden tot een verslechtering van de conservering.

BARANČIC, FT. and VL. SĚVČIK, Das Wirtschaftseigene Futter *12, 1966 (3)* 286–293.

VI

Het is niet met zekerheid vastgesteld, dat het ongeboren kalf de primaire oorzaak is van de dalende koperreserve van de drachtige koe.

GRIFT, J. v. d., Diss. Utrecht 1955

VII

De invloed van melasse op het gistingsproces in silage is beter dan die van een equivalente hoeveelheid suiker.

VERHOEVEN, W., Diss. Delft 1952
WIERINGA, G. W., Neth. J. Agr. Sci. *6* (1958) 204–210
WIERINGA, G. W., Proc. Xth Grassl. Congress. Helsinki 1966, 537

G. W. WIERINGA
Wageningen, 24 febr. 1967

VIII

Ter onderscheiding van boterzuurbacteriën die het kaasgebrek „laat los" veroorzaken van die, welke deze eigenschap missen is de zouttolerantie een beter criterium dan het lactaat vergistend vermogen.

GOUDKOV, A. V. and M. E. SHARPE, J. Appl. Bact. *28* (1965) (1) 63–73
GIBSON, T., J. Appl. Bact. *28* (1965) (1) 56–62

IX

Het is beter om, bij de definiëring van de gewenste chemische samenstelling van gras, niet slechts plantenfysiologische, maar ook dierfysiologische criteria aan te leggen.

WIT, C. T., W. DIJKSHOORN. J. C. NOGGLE, V.L.O. 69. *15*, 1–68.

X

Bij het kweken van betere roggerassen zal het streven naar een laag gehalte aan alkylresorcinolen van ondergeschikte betekenis dienen te zijn.

XI

Het is niet met zekerheid vastgesteld dat rogge een bacterieremstof bevat.

EL SHAMMA, Z. A., Z. blatt. f. Bakt., I. Abt., Orig. Bd. 183, (1961) 527–540.

XII

Het door verschillende wetenschappelijke instellingen verrichten van onderzoek aan eenzelfde probleem wordt veelal ten onrechte aangeduid als „dupliceering van onderzoek".

ACKNOWLEDGEMENT

The present investigation is a part of a study carried out bij the Institute for Storage and Processing of Agricultural Produce, Wageningen, with financial support of the Netherlands Grain Centre.

The feeding experiments with rats, an integral part of this work, have been carried out bij the Netherlands Institute of Nutrition, Wageningen. The author is much indebted to Prof. C. den Hartog, Director of the Institute, Dr. G. Pol, Deputy Director and Mr. P. van Kleef for their valuable advice, stimulating criticisms and their never lasting enthousiasm in the course of this investigation. Special thanks are due to Miss G. S. van Schaïk and Mr. J. Ruisch for their excellent technical assistance.

CONTENTS

INTRODUCTION

The production of rye in the Netherlands is restricted to the dry and sandy soils. Although the yield of rye (approximately 3000 kg/ha) in comparison to other grain crops is rather low, rye still plays an important part in the crop rotation on the sandy soils. From 1958 to 1963 the total rye production in the Netherlands decreased from 428.000 tons to 313.000 tons. In the same period the import of rye increased from 107.000–270.000 tons, thus keeping the total amount of available rye up to the level.

Of the total amount of 535.000 tons in 1958 approximately 85.000 tons were used for human consumption, 420.000 tons were available for animal feeding and 30.000 tons for other purposes. Only about one third of the feed grade rye was marketed. Considering that 4 million tons of concentrates were produced in that year, it can be calculated that the average percentage of rye in the commercially available concentrates was less than 5%. A percentage which since did not increase considerably.

The main cereals used in concentrates are maize, barley and oats. Compared to these as regards starch equivalents (SE) and digestible crude protein, rye can rank with the best; but contrary to expectations the demand for rye is low, because it is reputed to contain a growth inhibiting factor. This bad reputation is not only the cause of lower prices, but is also responsible for the fact that a considerable part of the total rye yield is not marketed at all, but has to be used for animal feeding purposes on the own farm. The combined effects of low prices and of undesirable surplus of rye in the rye producing areas contribute to the lower economic status of agriculture on the dry sandy soils.

Based on the results of German investigations on differences in harmfulness between rye varieties, it was thought that it might be possible to breed harmless rye varieties. But this was frustrated by the fact that large amounts of rye are necessary to test the effect of a rye variety on experimental animals. Thus it was concluded that, in search for better varieties, a chemical method, enabling the determination of the growth inhibitor in minute rye samples, was a prime necessity.

It is the aim of the present investigation to isolate the growth inhibiting factor and to develop an analytical method to determine this substance in micro quantities. Because during this work experimental animals were necessary for analytical purposes, some observations could be made on the nature of the effect of the factor on these animals.

CHAPTER I

SURVEY OF THE LITERATURE ON THE EFFECT OF RYE FEEDING ON ANIMALS

In 1900 VON KNIERIEM reported that rye, when fed in large amounts to cattle, sheep, horses, pigs, poultry or rabbits, spoiled the appetite, thus leading to lower growth results. Of the symptoms caused by rye feeding, the appetite spoiling action – or intake inhibiting effect – is mentioned in nearly all publications on the effect of rye feeding on pigs and poultry (RICHTER and coworkers (1930), HORN and PREIS (1930), HONCAMP (1932). Besides this effect, it is said in many German publications that pigs fed with rye become 'thickblooded' (German: 'dickblutig') (KELLNER 1905). This means that after slaughtering the animals bleed less completely. In the Netherlands it is said that overfeeding of piglets with rye causes red and lateron black spots on the skin, called 'branderigheid' (BROEKHUIZEN 1954). As to how far these symptoms can be identified with the 'thickbloodiness' and/or are exclusively caused by rye is not known. WEISER and ZAITSCHEK (1933), comparing rye feeding to wheat feeding, concluded that the former increased the heatfrequency of pigs. HORN and PREIS (1931) attributed the decreased growth of their rye fed pigs to the lowering of the food intake during the frequently occuring periods of heat. It should be mentioned however, that these two investigations were carried out with a few animals only and that the results obtained were not confirmed by others. Without overlooking the seldom encountered symptoms like a spotty skin, „thickbloodedness' and abnormal heat, it may be concluded that a lowering of the food intake and growth rate are the main symptoms of overfeeding with rye.

DAMMERS (1956) and VOOGD (1961), reviewing the literature on the effect of rye feeding on pigs respectively pigs and poultry, conclude that it is rather confusing. Although most authors agree that rations for pigs above 50 kg may contain 50% rye and for poultry 20% rye without measurable effects, higher percentages of rye are found to be harmful by some authors and innocuous by others. A critical survey of the literature shows that a number of causes can be given for this lack in unanimity. Differences in composition and feeding value of the diets to be compared, differences in the age of the experimental animals and differences in methods used to calculate the effect of rye, are responsible for the apparent differences in results.

Nearly all experimental evidence on the intake inhibitory effects of rye has been obtained by means of rations in which barley, maize or oats are substituted by rye. In attempting to assess the quantitative effect of rye on the production it must be kept in mind that the differences obtained will not only be due to a possible harmful factor in the rye, but that differences in feeding value may also play a part.

From the figures given in table 1 it can be concluded that rye and barley, as to starch equivalents and digestible crude protein, take up an intermediate position between maize and oats.

TABLE 1. Starch equivalents (SE) and digestible crude protein (dcp) values in some feedstuffs (CVB 1960)

feedstuff	SE	dcp in %
rye	70	7,9
barley	72	7,4
oats	68	9,3
maize	80	7,1
potato flakes	64	3,6

In a statistical study, CRAMPTON (1933) calculated for a number of experiments, carried out by others mainly in the USA and Canada, that the daily gain in weight of the animals, receiving rations with appr. 70% rye, was 6% lower than of the barley fed animals. The results obtained by HONCAMP (1932) who summarized 10 identical feeding trials carried out by 10 research stations in Germany were similar. The average daily intake of the animals on a diet containing 67% rye, 23% barley and 10% fishmeal was lower than the intake on 90% barley and 10% fishmeal. The daily weight gain of the rye-fed animals was 12% lower, whereas the feed conversion was 9% higher than of the controls. The addition of 10 or 20% molasses to the rye ration (thus reducing the rye percentage to 60% resp. 53%) could reduce the growth depression to 4 resp. 5%.

With rations containing up to 50% rye WILKENS (1930) found that the growth of the rye fed pigs was 8% lower than the growth of the barley- or maize-fed animals. This growth depression could be counteracted by the addition of 2% animal protein to the ration. SCHMIDT and VOGEL (1931) could not establish any harmful effect of rye on pigs. It should be mentioned however, that the rye ration contained 2% more fishmeal than the control ration containing barley. Instead of animal protein RICHTER *et al* (1961) used soybeanmeal and found that no growth inhibiting effect occurred when 50% barley was substituted by 48% rye and 2% soybeanmeal. In other experiments (RICHTER and ANTONI, 1960) the animals had free access to either ground barley or a mixture of ground rye and barley (50/50) after the consumption of a measured amount of a basic feed containing barley, soybean- and fishmeal and vitamins. Although the free intake of the rye/barley mixture was somewhat lower than that of barley the growth was not significantly lower. It may be assumed that the relatively higher protein level in the ration of the rye-fed animals played a part in masking the growth inhibiting effect of rye.

In attempts to evaluate the single feeds of a ration CRAMPTON (1933) encountered another problem. Using the system of ad libitum feeding of two different feeds, the substitution of one grain by another may be accompanied by a lower or higher intake of the other ingredients of the ration. CRAMPTON states that: 'although in this case the difference in the energy value of the total ration may not be exactly proportional to the energy difference of the substituted grain, the changes nevertheless are to be considered as effects of the grain substitution'. It may be true that such a feeding system comes near to practice, but it makes the quantitative evaluation on account of a harmful effect, other than through

unpalatability, a difficult task. The results of the feeding trials conducted by WILSON and WRIGHT (1932) form a typical example of the difficulties encountered when uncomparable rations are to be compared. Although the authors conclude that in comparison to maize and barley, rye is a relatively poor grain to fatten pigs on, the differences in weight gain are partly due to differences in dcp- and SE-values in the total rations.

Seen from an economical point of view rye can compete successfully with barley and maize if the rations for pigs above 50 kg do not contain more than 50–70% rye (WILKENS, 1930; RICHTER *et al*, 1930, 1931, 1959, 1960; HONCAMP, 1932). This conclusion is based on the observed intake and growth depression of 5–10% and on the fact that the price of rye is approx. 10% lower than that of barley and maize. However, on account of the lower daily gains, fattening pigs to marketing weight, requires more time.

It is generally accepted that young animals are more susceptible to the growth inhibiting effect of rye than older pigs. In the Netherlands it is advised that the maximum amounts of rye should vary from zero for piglets and sows, to 15% rye for pigs between 30 and 50 kg and 30% rye in the rations of older pigs (DE BOER 1958).

CRAMPTON (1936) who surveyed the literature before 1936 concluded that rye is less palatable for chicks and layers than wheat, maize or barley. No more than 20–30% rye should be given to poultry. This agrees perfectly with the results obtained by HELDER (1957); HELDER and VAN ALBADA (1956), while others concluded that 40–50% of the ration may consist of rye (HALPIN a.o. and DELWICHE 1940). In connection herewith it is of interest that already in 1936 CRAMPTON noted: 'that there is a wide variability of results, not only between different rations but also between successive tests conducted at the same station'. Differences in harmfulness between the different lots of rye, as well as differences in feeding value of the rations may play a part here.

Experiments on the effect of different rye varieties have been conducted by STAHL *et al* (1933) who found that the growth of pigs fed on imported La Plata rye was slower than on German rye. DAMMERS (1956, 1959), IWEMA and DE BOER (1957) and VAN WIERINGEN (1958), working with pigs and rats, concluded that better growth results were obtained with Petkuser winter rye as compared to tetraploid varieties. Among a number of inbred rye varieties tested, some showed a lower harmfulness than Petkuser. Although only a few of these strains were fed to pigs and rats, both showed the same response. HELDER and VAN ALBADA (1956) could not establish significant differences in palatability between rye varieties in experiments with laying hens.

As, however, the rye intake in this experiment was lower than the advised safe level of 20%, differences could hardly be expected. Although the aforementioned feeding experiments with pigs and rats have shown differences in harmfulness between varieties, the genetical nature of these differences has not been proved. In this connection it may be mentioned that DAMMERS (1959) found indications that other factors like soil type, fertilization and weather probably influence the harmfulness of rye.

The growth inhibiting effect of rye is often ascribed to the occurrence of ergot (Claviceps purpurea). According to JOHNSON and PALMER (1935) rations with toxic amounts of ergot (1 %) are 'so unpalatable that the animals refuse to eat it'. It will be clear that the effect of ergot must not be mistaken for the effect of rye itself. Data on the ergot content of the rations used in feeding trials are very scarce. Anyhow at the moment the ergot level in Western Europe lies far below 0,1 %, and the greater part of the experiments conducted by DAMMERS and VAN WIERINGEN (pers. comm.) is conducted with rye practically devoid of ergot contamination. Other authors attribute the poor results obtained with rye feeding to moulded or heated rye or to sprouting. RICHTER *et al* (1961) could not establish differences in weightgain between pigs fed with normal and pigs fed with sprouted rye, provided that the sprouted grain was not spoiled by moulds or bacteria. According to DAMMERS (1956) the observations of WEISER and ZAITSCHEK (1933) and of HORN and PREIS (1931) on the estrogenic activity of rye, are supported by the finding of SCHOOP and KLETTE (1955) that rye bran contains estrogenic substances. As however the estrogenic activity of rye bran was even lower than that of commercially available concentrates (DLG-mixture, sugar beet pulp, and other basic feedstuffs), it may be accepted that the eventual estrogenic activity of rye was not responsible for the decreased growth rate of rye-fed animals.

From nearly all feeding experiments showing the growth inhibiting effect of rye, it can be concluded that the diminished growth rate is caused primarily by a decrease of the intake, for which the bitter taste of rye is held responsible. Although HONCAMP *et al* (1932) state that the addition of molasses masks the bitter taste of rye, they erroneously conclude that sugar counteracts the intake inhibiting effect. The higher growth rate on the rations with molasses is probably due to the lower rye content of these rations. Sugar could not mitigate the ill effects of rye when fed to rats (VAN WIERINGEN, 1957).

The question is whether the lowering of the intake is caused by a bitter or otherwise disagreeable taste, or by any unknown physiological effect on the animal body. Literature on this problem is scarce and incoherent. VON KNIERIEM (1900) ascribed the lower growth rate to the lower digestibility of rye fat. It may be questioned however, whether the lower digestibility of as little as 1,5 % rye fat can account for a growth depression of about 10 %. Comparing rye feeding with the feeding of rations in which rye was substituted by animal protein and e.g. wheat, BICKEL (1939) found higher liver glycogen levels in the rye-fed animals. Storage of the rye decreased this glycogenic effect. Probably an insufficiency of one or more essential aminoacids in the rye containing rations was responsible for the increased storage of liver glycogen. As far as can be concluded from this work no relationship exists between this phenomenon and the growth- (intake-) inhibiting effect of rye. In 1957 VAN WIERINGEN conducted a number of paired feeding trials with rats on diets with 90 % rye or barley and 10 % caseine. When the intake of the 'barley' animals was restricted to that of the 'rye' animals growth was equal for both groups. Thus, no differences in digestibility were observed. When after about 3 weeks all animals received the

'barley' ration ad libitum 6 out of the 9 'rye' animals failed to eat immediately as much of the barley diet as did the 'barley' animals. From this observed aftereffect it can be concluded that the inhibition of the food consumption is not caused by a bitter taste but by a, still unknown, harmful effect on the constitution of the animals.

To locate the harmful factor, DAMMERS (1954, 1955) conducted feeding experiments with pigs, with rations containing high amounts of rye flour, -bran, and -grit in comparison with barley and barley millery fractions. Although on the rations containing rye growth rates were slower than on the barley rations, the results showed clearly that rye bran and -grit, were more harmful than the rye flour.

For long it has been common knowledge, that in practice more difficulties are encountered in feeding freshly harvested rye, as compared to feeding rye that had been stored for some months (POTT, 1907; BICKEL, 1939). POTT and POPP (1936) advice to boil or steam rye prior to feeding it to livestock. This procedure was used to a great extent in previous years, but is nowadays considered too expensive. Moreover the value of this steaming is doubtful since DAMMERS (1955) showed that a part of the steamed rye kernels was not digested by pigs. Better results were obtained by boiling milled rye, and DAMMERS concluded from this that the toxic factor was destroyed or volatilized during the heat treatment. In accordance with this POPP (1936) found that better growth results were obtained with rye flakes as compared to rye meal. Other feeding experiments, however, showed that rye flakes were inferior to untreated rye (DAMMERS 1956; MARX *et al* 1962). Because a comparison between the different drying methods used, is not possible, no explanation can be given for the lack of unanimity in the conclusions. It may be assumed, however, that aminoacid losses, occurring during the drying process, are responsible for the poor results obtained with flakes. Although from the work of DAMMERS it seems that a steam treatment improves the quality of rye, from an economical point of view heat treatment and the production of flakes can hardly be expected to be warranted (MARX *et al*).

CHAPTER II

PRELIMINARY EXPERIMENTS ON THE EFFECTS OF RYE AND RYE FRACTIONS ON RATS

1. Introduction

Judging from the literature cited in chapter I it will be clear that different species of animals are susceptible to overfeeding with rye, with, as the main symptoms, a decrease of appetite and growth. On the assumption that an unknown chemical substance, occurring in the rye, was responsible for this effect, it was thought desirable to elucidate the structure of this compound and to develop a method to estimate the harmfulness of different rye varieties. Pending the chemical analysis method, however, the animal experiment is the only means to determine this growth inhibiting effect.

The experiments described in this chapter are mainly based on the results obtained by Dammers and Van Wieringen (see chapter I). Considering the necessity of concentrating the growth inhibiting factor by separating the rye in harmful and harmless fractions, separating methods like destillation and extraction were included in the first experiments.

2. Materials and methods

Rye and barley

In September 1961 2 lots of 'Petkuser' winterrye (lot A combine-harvested and lot B threshed after field curing) and one lot of winter barley of unknown origin, all of these harvested in 1961 were treated in different ways. Data on the composition of the treated materials are given in table 2.

Table 2. Chemical composition of the basic feedstuffs

		in % of dry matter				
grain	treatment or fraction	dry matter in %	crude protein	crude fibre	ash	fraction in % of total grain
rye (A)	–	85,9	11,9	2,2	2,1	100
rye (A)	steamed	88,8	11,9	2,0	2,1	–
rye (A)	bran and grit	86,4	15,5	3,6	3,0	68,6
rye (A)	id. steamed	88,4	15,4	3,8	2,9	–
rye (A)	id. extracted	87,4	15,8	3,5	3,0	67,1
rye (B)	bran and grit	85,9	14,7	3,6	3,0	68,8
barley	bran and grit	86,4	14,5	7,9	3,2	86,4

Milling

For the first experiment the grain was milled on a flour mill* and the bran and grit fractions of each lot were put together, and passed through a 'Pepping' laboratory mill with 2 mm sieve. For the second experiment whole rye (A) and steamed whole rye (A) were passed through the 'Pepping' mill.

Steaming

Rye (A) and the combined fractions of bran and grit of rye (A) were subjected to a steam treatment at 100–110° C during 5 hours and dried afterwards in vacuum at 80° C. This treatment yielded 500 ml destillate/kg raw material, with a chemical oxygen demand (COD) of 350 and 342 mg/l respectively.

Extraction

Rye (A) -bran and -grit were extracted for 8 hours in a 5 l Soxhlet apparatus with light petrol (bp $<$ 40° C). The residue was vacuum dried overnight at 80° C. Solvents were removed from the oil fraction in a rotating film evaporator at 80° C at reduced pressure.

Experimental animals and diets

Male rats of the white Wistar strain were used as experimental animals. To obtain animals of comparable weight, a large number of females was mated within one week. The litters were reduced to 7 newborns, discarding the female ones 2 days after birth. The experimental groups were composed at weaning, dividing the male littermates over the groups. Each group was composed of 9–10 animals of approximately the same average body weight. The animals were housed individually in cages. Food and water were supplied ad libitum. Food consumption was measured daily and body weight once a week.

To investigate, in the first experiment the influence of:

a. different harvesting methods
b. steaming and
c. petrol ether extraction on the growth of rats,

8 groups of 9 rats were used.

The diets were composed in such a way that the energetic value of all diets was approximately the same. Calculated on dry matter they contained 13,1% crude protein, 73,0% carbohydrates and 7,1% crude fibre. The fat content of diet IV was about 1,5% lower than that of the other diets, because no compensation was provided for the extracted rye oil. Due to the rather low biologic value of the grain protein, the protein content of these diets was sub optimal.

In feeding experiment no. 2 rye meal was used instead of bran and grit and by adding 2% casein, the protein content was brought up to 12,6%. The composition of the diets is given in the tables 3 and 4.

* Thanks are due here to the 'Experimental Station of Milling and Baking' Wageningen for the milling of the rye and barley used for this experiment.

TABLE 3. Composition of the diets of experiment no. 1 in g

ingredients	diet number							
	I	II	III	IV	V	VI	VII	VIII
rye bran and grit								
A	933	–	–	–	933	933	–	–
B	–	922	–	–	–	–	–	–
A steamed	–	–	919	–	–	–	–	–
A defatted	–	–	–	906	–	–	–	–
destillate (A)	–	–	–	–	1000	–	–	1000
crude oil (A)	–	–	–	–	–	20	–	–
barley bran and grit	–	–	–	–	–	–	1000	1000
cellulose	39	37	37	40	39	39	–	–
dextrose	44	–	40	57	44	44	25	25
maize oil	60	60	60	60	60	40	60	60
vitamin-salt mixture*	30	30	30	30	30	30	30	30
water	1013	1000	1033	1016	13	1013	1004	4

* Composition of the vitamin salt mixture

$CaHPO_4$	60 g	$FeSO_4$	750 mg	NaF	25 mg
KCl	30 g	$Al_2(SO_4)_3$	500 mg	$CoSO_4$	12 mg
Na_2HPO_4	24 g	$ZnSO_4$	300 mg	Na molybdate	12 mg
$MgCO_3$	6 g	$MnSO_4$	75 mg	Boric acid	12 mg
Na-citrate	3 g	$CuSO_4$	75 mg	KJ	1 mg
		KBr	60 mg	As_2O_3	0,5 mg
		$NiSO_4$	25 mg		

To 30 g of this mixture was added:

vitamin			
B_1	6 mg	Choline	300 mg
B_2	3 mg	Inositol	300 mg
B_6	3 mg	P. aminobenzoic acid	100 mg
Ca pantothenate	15 mg	folic acid	1 mg
Nicotinamide	25 mg	B_{12}	0,5 mg

vitamin A and D were administered 3 times per week by pipetting 0,1 ml. ground nut oil containing 200 mg vitamin A acetate and 0,67 mg vitamin D_3 per 100 ml.

TABLE 4. Composition of the diets of experiment nr. 2 in g

	A	B	C	D
rye	900	900	900	–
rye steamed	–	–	–	873
rye destillate	–	1000	–	–
rye oil	–	–	40	–
casein	20	20	20	20
maize oil	60	60	20	60
vitamin salt mixture*	30	30	30	30
water	1000	–	1000	1027

* see table 3

3. Results

From the figures in table 5 it can be seen that food consumption, growth and food efficiency ratio (f.e.r.) for the groups of animals on diets nrs I and II were nearly the same. Thus no difference in harmfulness could be observed between the lots of rye, which were harvested in different ways.

Table 5. Exp. nr. 1: Average food consumption, growth and food efficiency ration over the 32 day period

	diet	consumption g	growth g	food efficiency ratio (growth/consumption)
I	rye A	382	78	0,202
II	rye B	394	79	0,200
III	rye A steamed	409	49	0,122
IV	rye A defatted	442	94	0,212
V	rye A + dest.	389	81	0,208
VI	rye A + rye oil	371	74	0,199
VII	barley	593	93	0,157
VIII	barley + dest.	568	90	0,158

Greater differences were found between some of the other groups. To judge the significance of these differences, Student's T test could not be used, due to the irregular distributions within the groups. For this reason the tables of critical values of the Wilcoxon rank sum test, as composed by Wabeke and Van Eeden (1955) were used (Wilcoxon, 1945).

Although the diets were composed in such a way that differences in metabolizable energy and digestible protein were as small as possible, significant differences in consumption, growth and f.e.r. were observed between the groups receiving 'barley' and those receiving 'rye'. No other explanation could be found for this, than that a part of the barley diet was spilled by the rats, which was probably caused by the more fibrous structure of the barley bran diets.

The fact that no significant differences occurred betweeen the growth results on the diets nrs VII and VIII indicates that the rye destillate diet did not exert any harmful effect on rats. Comparing the diets nrs I and V this conclusion could be confirmed. In contradiction to the results obtained by Dammers (see chapter I), steaming led to a higher intake but to a significantly lower growth and f.e.r. ($2\,P = 0{,}001$). The high steaming temperature and the resulting brown colour of the steamed rye bran did anticipate Maillard reactions. Pol (1960) was able to show that steaming of the rye bran resulted in a considerable loss of lysine. Knowing that the protein content of the diets was limited and that lysine is the limiting amino acid of the grain proteins the poor growth result obtained with the steamed rye diet becomes understandable.

Comparing the diets nrs IV, I and VI it will be seen that in this sequence the consumption of the diets decreased. In accordance with this, growth and f.e.r. decreased in the same order. The differences between IV and I and between I and V did not reach the significance level of 5%.

The difference between diet nr. IV (extracted rye) and nr. VI (rye with added rye oil) were however significant, the 2 P levels being 0,014, 0,002 and 0,035 respectively for consumption, growth and food efficiency ratio. The results of experiment nr. 2 (table 6) confirmed those of the first.

TABLE 6. Exp. nr. 2: Food consumption, growth and food efficiency ratio (39 days) and 2 P values in respect of diet I

diet	food consumption		growth		f.e.r.	
	g	2P-value	g	2P-value		2P-value
A rye	731	–	168	–	0,229	–
B rye + dest.	760	0,12	178	0,28	0,234	0,20
C rye + rye oil	656	0,06	134	0,001	0,204	0,0002
D steamed rye	764	0,24	151	0,035	0,197	0,0003

Again no significant differences were found between the A-rye diet and B-diet with added rye destillate. The animals on diet D (steamed rye) consumed insignificantly more food than the animals on diet A, but the growth was significantly lower, and the difference in f.e.r.'s was even highly significant. The rye oil caused a lowering of the food consumption (nearly significant) as well as of the growth and the food efficiency ratio (both highly significant).

4. DISCUSSION AND CONCLUSIONS

The aim of the first experiments was to find out whether the rye factor was either steam volatile, destructed by heat or light petrol soluble. Rye destillates added to rye bran – barley bran – and rye diets did not lead to a significant decrease of growth. From this it was concluded that the destillate did not contain any harmful substance.

Less clear were the results of the heat treatment on the rye factor. The detrimental effect of heating on the lysine content, and perhaps also on the digestibility of other compounds, obscured the effect of a possible breakdown of the rye factor. Thus no conclusion on the heat resistance of the rye factor could be drawn from these experiments.

The oil fraction had a distinct influence on food consumption and growth. Especially as compared to the growth promoting effect of the fat extraction process, the lower growth and food consumption of the diets containing rye oil lead to the conclusion that the rye factor was soluble in light petrol.

CHAPTER III

FURTHER EXPERIMENTS ON THE GROWTH INHIBITING EFFECT OF RYE OIL

1. Introduction

In the feeding trials described in chapter II the materials to be tested formed a considerable but varying part of the energy- and protein value of the diets. Although the diets were composed in such a manner that the chemical composition with regard to energy value and protein content of all the diets to be compared was nearly the same, differences in digestibility could not be avoided. The finding that a growth inhibiting factor could be concentrated in the 1,2–1,3% rye oil fraction, made it possible to enhance the setup of the feeding trials. Therefore it was thought desirable to repeat the feeding experiments on the effect of the rye oil fraction, with reproduceable well defined diets without any possible interference of other rye components.

Another reason to subject the rye oil to further examination was the fact, that the extracted oil fraction smelled distinctly of petrol. Even a vacuum destillation at 140° C was not sufficient to remove organoleptic perceptible residues of the light petrol used.

Because for technical reasons no use could be made of analytical grade light petrol, the hazard of incorporating toxic cyclic substances in the extract could not be excluded. Hence it was deemed necessary to investigate the influence of solvent residues on the effect of the rye oil fraction.

2. Experiments on the possible effects of solvent residues

The effect of light petrol residues

To test the influence of solvent residues, light petrol extracts of rye and barley were produced according to the procedure described in chapter II. The intake and growth inhibiting effect of these oil fractions was investigated in a feeding trial with rats. The basic diet was composed as follows:

potato protein	20%	saccharose	6%
potato meal	65%	salts and vitamins (chapter II, table 3)	4%
potato fibre	1%	maize oil	4%
		vitamins A and D (see table 3).	

For the experimental groups, the maize oil in this diet was replaced by rye oil or barley oil. Based on the fact that the iodine numbers of rye (123) and barley (113) were comparable to those of maize oil (117) this substitution was considered permissable.

In order to obtain the certainty that eventual differences in growth were caused by solvent residues and not by differences in feeding value between the

extracted oils and the pressed and refined maize oil a fourth group was included in this trial. This group received maize oil, recovered after dissolving it in 3 parts of light petrol.

The feeding experiment lasted 5 weeks. During this time in all groups a number of animals suffered from diarrhoea, and in each group one animal died except for the rye oil group which suffered 3 deaths. Average figures for food consumption and growth of the healthy animals are given in table 7.

TABLE 7. Exp. nr. 3: Consumption and growth of rats on diets with maize, rye and barley oil

group no.	diet	number of healthy animals	grams/animal/5 weeks	
			food consumption	growth
I	maize oil 4%	6	480	104
II	maize-oil[1] 4%	5	470	89
III	rye oil[2] 4%	6	390	72
IV	barley oil[2] 4%	7	435	86

[1]) light petrol treated [2]) light petrol extracted

Consumption – and growth figures of the rats on diet nr. I were highest. Compared with this the consumption of the animals on diets nrs III and IV was significantly lower (2 P resp. 0,008 and 0,03). The consumption figure on diet II was not significantly lower (2 P = 0,82), but growth and food efficiency ratio figures on diet nr. II were indeed significantly lower (2 P = 0,01). These results strongly indicated the occurrence of a harmful effect of the light petrol, disturbing the effect of the rye oil.

The effect of acetone extraction

To avoid the difficulty of the toxic –, or at least the growth inhibiting effect of petrol residues, some other solvents were tested. Ethanol, acetone and chloroform extraction were carried out and the oil yields were compared with those of the light grade petrol extraction. It appeared that alcohol extraction of rye grit gave by far the highest yield with 6,7%, whereas chloroform and acetone both yielded 2,5% oil and light petrol 1,5%. In table 8 the oil yields of subsequent solvent extractions are given. From the figures it can be concluded that acetone, together with some other substances, extracted all the petrol extractable material.

Similar results were obtained with chloroform instead of acetone. Because the hazard of chloroform residues in feeding trials was considered far greater than of acetone residues, acetone extraction was preferred. For feeding trial nr. 4 rye and barley were extracted with acetone in a 5 l Soxhlet-apparatus for 8 hrs. During the evaporation of acetone from the oil the extract separated in an oil layer and a water layer. After freezing, the oil layer was scraped off and freed of acetone residues in a rotating vacuum evaporator at max. 100° C. The acetone

TABLE 8. Oil yields after subsequent extractions with different solvents

solvent		oil yield in %				
1st	2nd	1st	+	2nd	=	total
light petrol	acetone	1,51		0,94		2,45
acetone	light petrol	2,50		0,04		2,54

residues were 0,2 and 0,3 % respectively. The water layer was concentrated to a syrupy brown substance in the same apparatus. Approximately 15 g of this substance was obtained per 100 g of oil. Analogous to the petrol treatment of maize oil in exp. nr. 3, maize oil was dissolved in acetone and the acetone evaporated in the same manner as used for the rye and barley oil.

Because the growth results on the basic diet used in exp. nr. 3 were not quite satisfactory the basic diet was altered as follows.

TABLE 9. Composition of the basic diet

potato protein	15%	maize oil	10%
caseine	5%	salts and vitamins (see table 3)	4%
potato meal	50%	vit. A-D mixture (see table 3)	

In a preliminary feeding trial preceding exp. nr. 4 this basic diet with untreated and with acetone treated maize oil was tested on 2 groups of 5 young rats. Although the acetone treated maize oil purposely contained 1,9 % acetone no ill effects whatever were observed, and both groups grew equally well. To intensify the growth inhibiting effect of the rye the percentage of the oil to be tested was increased from 4 to 10 %. As can be seen from the results in table 10 this brought on the death of 9 of the 10 'rye oil' rats within 17 days. The only surviving animal was changed over to diet nr. I and showed a quick recovery.

TABLE 10. Exp. nr. 4: Influence of acetone extracted rye and barley oil on the growth of weanling male rats

diet		after 10 days			after 17 days		
nr.	oil source	number surviving animals	consumption g	growth g	number surviving animals	consumption g	growth g
I	maize	10	104	36	10	202	63
II	maize[1])	10	103	33	10	198	57
III	rye[2])	8	51	–	(1)	–	–
IV	barley[2])	10	100	31	10	200	62

[1]) acetone treated [2]) acetone extracted

With regard to consumption – and growth figures no differences were observed between diets nrs I, II and IV. It was thus concluded that the growth – and

intake inhibiting effect of diet nr. III was exclusively caused by the rye oil and not by any experimental artifact.

Although the occurrence of a growth inhibiting factor in the water phase, obtained during evaporation of the acetone extract from rye, was not anticipated the effect of this rye fraction was tested, in comparison to the corresponding barley fraction. The feeding trial was carried out with 2 groups of 10 male weanling rats of the Wistar strain. The experimental diets contained 15 g concentrated 'water phase' per kg basic diet (table 9).

Because a part of the 'water phase' obtained from barley was spilled, the 'barley' group received the basic diet during the last 13 days of the experiment. The results are given in table 11.

TABLE 11. Exp. nr. 5: Influence of the acetone extracted 'water phase', derived from rye and barley, on consumption and growth of rats in grams

time in days	diet with:			
	rye		barley	
	consumption	growth	consumption	growth
day 1–17	173	48	175	50
			basic diet	
day 18–29	162	40	151	37
total: 29 days	335	88	326	87

The differences in intake – and growth figures between both groups were insignificantly small. It can be concluded therefore that the rye factor was exclusively concentrated in the acetone extracted rye oil.

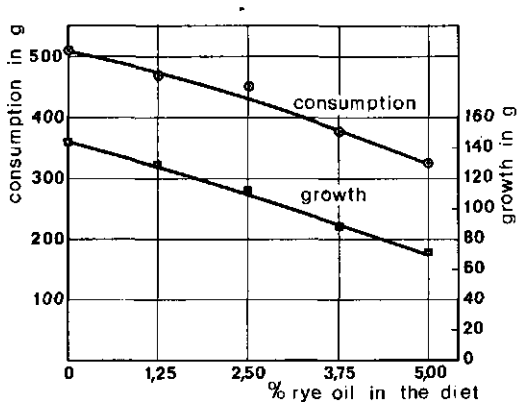
3. THE QUANTITATIVE EFFECT OF ACETONE EXTRACTED RYE OIL ON THE GROWTH OF RATS

In the first experiments low doses of rye oil were tested on rats. This led to small differences in food consumption and growth of the animals, and in some instances the statistical significance of these differences was questionable. With intent to achieve a greater and more significant response, the rye oil content was increased to 10% in exp. nr. 4. Unfortunately this resulted in the death of the majority of the rats. No doubt these lethal doses form the most irrefutable proof of the toxicity of the substance under investigation. The administration of sublethal doses however is to be preferred because food consumption and growth figures enable the quantification of the harmful effect.

In order to investigate the influence of different doses of rye oil, feeding trial nr. 6 was conducted with 5 groups each of 10 young male rats. The diets were composed of the basic diet given in table 9 in which 0%, 1,25% 2,50%, 3,75% and 5% maize oil respectively had been replaced by acetone extracted rye grit oil.

The results plotted in fig. 1, show a nearly rectilinear relation between the

FIG. 1. (Exp. 6) Influence of the rye oil content of the diet on the growth of rats during 5 weeks.



percentage of rye oil in the diet and growth and food consumption figures of the rats. The significance of the differences between the bodyweights of the animals is calculated according to the Wilcoxon test and the 2P values are given in table 12.

TABLE 12. Exp. nr. 6: Significance levels (2 P) of the differences in bodyweight between the 5 groups

diet with percentage rye oil			2 P
0,00	versus	1,25	0,04
1,25	versus	2,50	0,04
2,50	versus	3,75	0,0002
3,75	versus	5,00	0,07

From these figures it can be concluded that all differences are significant except for that between the animals receiving 3,75% and 5% rye oil. In this case the lower significance was caused mainly by the fact that 4 animals of the 5% rye oil group died during the experiment.

CHAPTER IV

EXPERIMENTS ON THE FRACTIONATION OF RYE OIL

1. THE SAPONIFICATION OF RYE OIL

In order to isolate the growth inhibitor it was attempted to concentrate the active substance in a smaller fraction. The first method used to achieve a more concentrated product was the saponification of the rye oil and the isolation of the fractions 'unsaponifiable matter' and 'fatty acids'. Considering that it would not be justified, while testing these fractions in a feeding trial, to compare the growth results of a diet with fatty acids with those of a diet with oil, it was decided to include the corresponding barley oil fractions in the experiment. Barley oil was chosen because the growth of rats on this oil was shown to be as good as on maize oil (see table 10).

For the saponification 15 grams of acetone extracted oil were boiled under reflux during 1 hr with 15 g NaOH anal. grade, 100 ml water and 40 ml ethanol. The soapstock was diluted with tenfold water and percolated 16–24 hrs with diethylether. The ether fraction subsequently was washed with water, with 0,1 N NaOH, and washed again with water till neutral to phenolphtalein. The soapstock was acidified to pH 2 with 9 N H_2SO_4 and the fatty acids were dissolved in diethylether. The fractions obtained, were freed of ether in the rotating vacuum evaporator. After evaporation of the ether a small portion of acetone was added and evaporated.

TABLE 13. Exp. nr. 7: Amounts of saponification products and oil in the diets

diet nr.	oil source and -fraction	yield %	'rye oil' added in % of diet	maize oil added in % of diet
I	rye oil	100	10	0
II	rye fatty acids	91	9	1
III	rye unsaponifiable	9	1	9
IV	maize oil	–	–	10
V	barley oil	100	10	0
VI	barley fatty acids	94	9	1
VII	barley unsaponifiable	6	1	9

The fractions were tested in feeding experiment nr. 7 (table 13 and 14). The basic diet given in table 9 was used. In this diet, the rye oil fractions to be tested were substituted for maize oil in amounts proportional to the percentage of the fraction occurring in the rye oil. The barley oil fractions were added in amounts equal to those of the corresponding rye oil fractions (see table 13). In this way a comparability is achieved between the different groups: Diet nr. III contains as much 'rye oil unsaponifiable' as Diet nr. I, and furthermore Diet nr. VII has a comparable amount of barley oil unsaponifiable.

TABLE 14. Exp. nr. 7: Influence of rye oil fractions on the average food consumption and growth of rats during 3 weeks

diet nr.	number of surviving animals	food consumption g	growth g
I	3	93	0,7
II	7	161	49
III	8	183	59
IV	20[1])	245	86
V	10	234	83
VI	10	204	74
VII	9	196	65

[1]) All groups originally contained 10 animals, except for the control group nr. IV with 20 animals

Figures on growth and food consumption of young rats during 3 weeks are given in table 14. In perfect accord with the results of exp. nr. 5 no significant differences showed up between the growth results on maize oil and barley oil (diets IV and V), whereas the growth on 10% rye oil (diet nr. I) was very poor and 7 animals died. In comparison with this the results of the other groups are less clear. Growth on the diets II and III (rye fatty acids resp. rye unsaponifiable) is significantly lower than on the control diet IV ($2\ P = 0{,}003$) but significantly better than on diet I ($2\ P = 0{,}003$). Peculiar is the development of the difference in growth between groups II and III: Although the growth on rye unsaponifiable after one week was significantly better ($2\ P = 0{,}009$); this significance decreased during the second and third week to $2\ P = 0{,}36$ and 0,68 respectively.

Another way to judge the results of the experiment is provided by the knowledge obtained from exp. nr. 6 (chapter III). The inversely rectilinear relationship between the growth of rats and the percentage of growth inhibiting factor in the diet enables grafical calculation of the amounts of harmful substance in the 2 fractions (fig. 2).

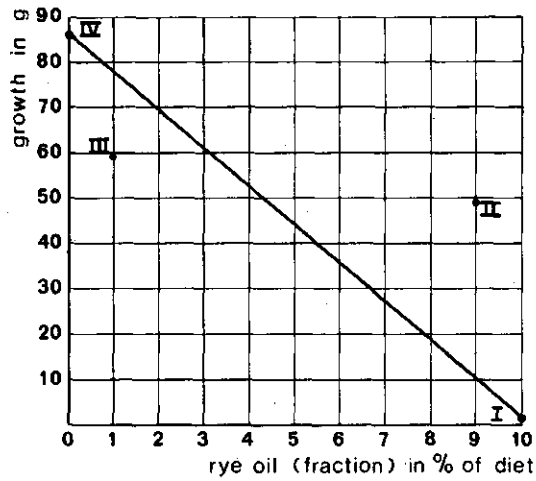


FIG. 2. (Exp. 7) Growth of rats on diets without rye oil (IV), with rye oil (I), rye oil fatty acids (II) and rye oil unsaponifiable (III).

From this graph it can be concluded that the growth inhibiting effect of rye fatty acids is lower than that of an equivalent amount of rye oil. In the same way it can be seen that 1% rye oil unsaponifiable is more harmful to rats than 1% rye oil. This tempts to the conclusion that the harmful factor, although not completely, migrated to the fraction 'unsaponifiable'. The validity of this reasoning is lowered however by the growth results of the rats which received the barley oil fractions.

In comparison to barley oil, the growth on diets with barley fatty acids and barley unsaponifiable were lower (2 P = 0,06 and 0,04). This indicates that during the preparation of the fractions a laboratory artifact was introduced in the experiment. Although this unexpected result did anticipate a similar effect to be incorporated in the rye oil fraction, correction of the figures for this effect was considered not justified. Comparison of the growth results on rye oil fractions to the corresponding barley oil fractions was the only way left to judge the distribution of the rye factor over the fractions obtained by saponification. The difference in growth between group II and VI in favour of the barley oil fatty acids was significant (2 P = 0,016). The difference between diets III and VII however could not reach the 5% probability level. This line of thought thus points to the occurrence of the rye factor in the fatty acids.

Although the disturbing side effect of saponification did not permit a definite conclusion, it will be clear that in the case of a complete migration of the rye factor to one of the saponification fractions the growth of rats on this fraction would at least have been as poor as on the rye oil.

Because this was not the case it can be concluded that the separating method used in exp. nr. 7 was of no use in concentrating further the rye factor.

2. Solvent fractionation of rye oil

Because exp. nr. 7 did not successfully lead to a separation of the rye oil in a harmful and a completely harmless fraction, this method of fractionation had to be abandoned. In view of the difficulties, encountered in interpreting the growth results of animals fed on oil fractions obtained by chemical treatments, a non-chemical fractionation seemed preferable. It was tried therefore to make use of the possible differences in solubility between the different rye oil components. The first attempts in this direction were the precipitation of part of the rye oil either after cooling or after addition of water to an acetone solution of the acetone extracted rye oil. Decreasing the temperature or increasing the water content of the solutions resulted in a gradual increase of the precipitate without reaching a separation in definable fractions. This, together with the concentration dependency of this procedure, made the usefulness of this method of separation questionable.

Because it was already known from the experiments described in chapter III (table 8) that extraction by acetone yielded more extract than extraction by light petrol, acetone extracted rye oil was mixed with 8 volumes warm light petrol (anal. grade, bp 35–40° C). After cooling to room temperature the mixture was

filtered, and the residue washed with cold light petrol. The final residue which amounted to 9% of the rye oil, was smaller than could be expected from the previous experiments.

After evaporation of the light petrol the petrol soluble part was mixed with 6 volumes of boiling methanol. The insoluble fraction was separated from the solution, the solution was cooled to 2° C and the resulting precipitate was filtered off. The quantitative results of this fractionation are given in table 15 (exp. nr. 8).

In experiment nr. 9 this fractionation was repeated with some alterations. The light petrol insoluble fraction was washed more thoroughly with warm light petrol resulting in a decrease of this fraction. Furthermore the methanol solution was cooled down to –10° C which led to a considerable increase of the precipitate (see table 15).

TABLE 15. Exp. nr. 8 and 9: Results of the solvent fractionation

rye oil fraction	fraction number	yield in %	
		experiment nr. 8	experiment nr. 9
light petrol soluble	2	91	97
light petrol insoluble	3	9	3
methanol insoluble (60° C)	5	32	29
methanol precipitate	6	8*)	26**)
methanol soluble	7	51*)	42**)

*) separated at + 2° C
**) separated at — 10° C

The fractions obtained were tested on rats in the same way as in the foregoing experiments. In the basic diet (table 9) rye oil was substituted for maize oil as outlined in table 16.

TABLE 16. Exp. nr. 8 and 9: Rye oil (fraction) in % of the diets

diet nr.	rye oil fraction nr.	rye oil (fraction) in %	
		exp. nr. 8	exp. nr. 9
I	rye oil	5	4,5
II	2	4,5	4,5
III	3	0,5	0,3
IV	2 + 3 = rye oil	5	4,5
V	5	1,5	1,5
VI	6	0,5	1,5
VII	7	2,5	1,5
VIII	5 + 6 + 7 = 2	4,5	4,5
X	2	–	1,5
XI	maize oil	–	8,5% maize oil
XII	maize oil	–	10% maize oil

As can be seen from table 16, in exp. nr. 8 the rye oil fractions were added to the diets in proportion to the yield of the fractions. Except for fraction 3, in experiment nr. 9 the separate fractions were not tested in amounts proportionate to the yield of the fraction, but in a fixed percentage of 1,5%. Thus the effect of the fractions 5, 6 and 7 could be compared with 1,5% rye oil (diet X) and fraction 2 with 4,5% rye oil (diet I). To test the possible side effects of the fractionation rye oil was reconstituted from fraction 2 and 3 and fraction 2 was reconstituted by joining the fractions 5, 6 and 7.

These reconstituted oils were tested in the diets IV and VIII. Besides the normal control diet XII with 10% maize oil, diet XI with 8,5 %maize oil and 1,5% agar agar was included in the experiment. In case one of the fractions 5, 6 or 7 would prove to be indigestible, diet XI offered the possibility to compare the growth on the diets with these fractions with a control diet with the same maize oil content.

The average weight of the animals at the beginning of the feeding trials was 47 g and 42 g in the exp. 8 and 9 respectively. The results of both feeding experiments are given in table 17.

TABLE 17. Exp. nr. 8 and 9: Influence of rye oil and rye oil fractions on growth of rats in g

diet	Experiment nr. 8			Experiment nr. 9		
	number of surviving animals	food consumption (8 weeks)	growth (8 weeks)	number of surviving animals	food consumption (5 weeks)	growth (5 weeks)
I	4	525	118	4	208	38
II	10	684	155	3	261	41
III	10	832	197	9	462	121
IV	6	591	131	6	270	52
V	10	873	204	8	463	139
VI	10	868	201	8	353	93
VII	10	703	161	10	366	98
VIII	7	722	161	8	276	63
X				10	429	119
XI				10	520	145
XII				10	558	121

At first sight the results of exp. nr. 8 show a significant difference in intake and growth between the animals of the groups I and II, I and III and II and III, but not between I and IV. It can be concluded that fraction 2 (the light petrol soluble material) contained more of the rye factor than the petrol insoluble fraction 3. The fact however that the growth on the reconstituted rye oil (diet IV) was lower than on diet II implicated that fraction 3 was not quite harmless. In comparison with the results of the petrol fractions, the effect of the three methanol fractions (5, 6 and 7) is more clear. The growth on the diets with the fractions 5 and 6 was significantly better than on fraction 2 from which they were derived. The growth

on fraction 7 however was not significantly different from the growth on fraction 2 nor from that on diet VIII with the reconstituted fraction 2. Thus it can be concluded that the total growth inhibiting effect of fraction 2 migrated to fraction 7 (the fraction soluble in methanol at 2° C).

Since the fractions were tested in different percentages, it is interesting to judge the growth differences of the rats in respect of the percentage of the fraction added to the diets. Although in exp. nr. 8 no control group was included, the growth of control animals was estimated with the help of the growth results of the control groups in the exp's 6 and 9. When these figures are corrected for the starting weight of the animals, a growth between 140 and 150 g is to be expected in 5 weeks. Hence a growth of 145 g was estimated for the growth of the lacking control group, and in fig. 3 straight lines are drawn between this point and the points I and II, representing the 5 weeks growth of the animals on the diets with rye oil and fraction 2 respectively. In this graph, the growth inhibiting effect of the diets II and III can be read on the lower line (C – I (IV)), and the effect of the diets V, VI and VII with the aid of the upper line (C – II (VIII)).

Although the arbitrary point C does not permit the quantitative determination of the growth inhibiting effect of the fractions it will be clear that fraction 3 contains relatively more of the inhibiting factor, whereas fraction 2 contains relatively less. This way of judging the growth results not only confirms the previous conclusion that fraction 3 is not quite harmless, but even indicates the enrichment of fraction 3 with the rye factor.

Judging the effect of the diets V, VI and VII in comparison to the theoretical growth line (C – II) in fig. 3 it is clear that fraction 7 contains most of the inhibiting factor, but that the fractions 5 and 6 may also contain a small amount. The fact, however that the growth on diet VIII with reconstituted fraction 2 exerts a growth inhibition not significantly different from that of fraction VII on

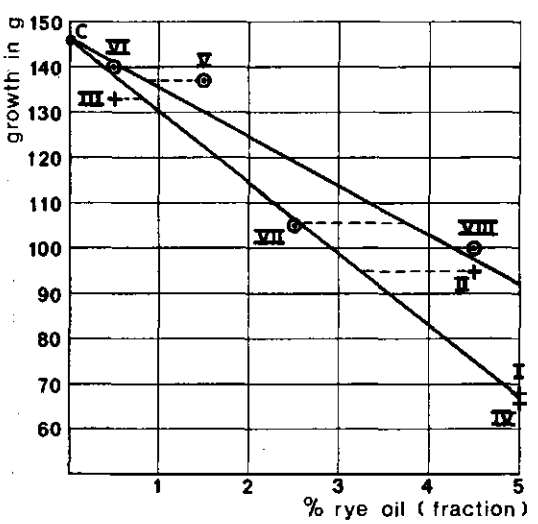


FIG. 3. (Exp. 8) Influence of rye oil and rye oil fractions on the growth of rats (5 weeks).

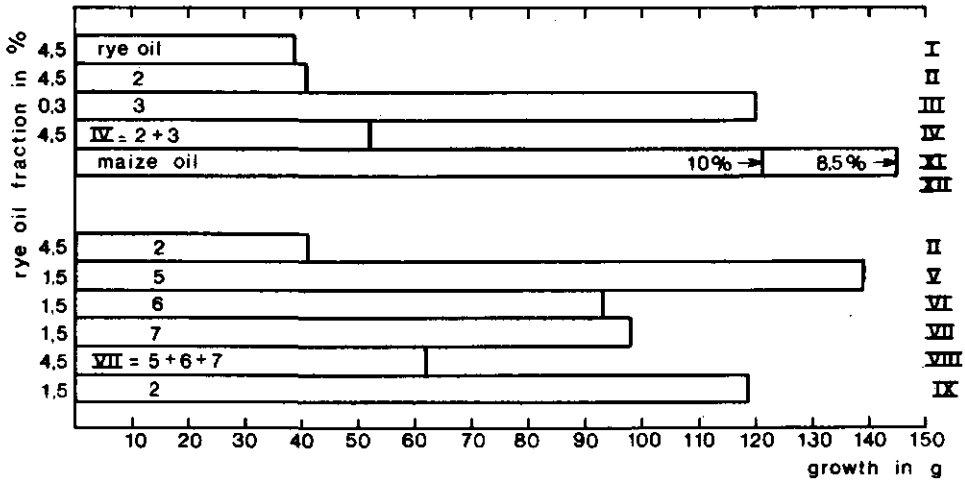


FIG. 4. (Exp. 9) Growth of rats on different rye oil fractions.

its own shows that it is justified to conclude that the growth inhibiting substance is soluble in methanol at 2° C.

Since in exp. 9 the fractions were not fed to the animals in proportion to the fraction yield, the method used in fig. 3 cannot be used in evaluating the results given in table 17. For better comparison the growth results of exp. 9 are plotted in fig. 4. Between the 2 control diets an incomprehensible difference in growth occurred in favour of diet XI. Although it was the intention to replace 1,5% maize oil in diet XI by an indifferent and undigestible substance, the substitution of agar for oil exerted an intake and growth promoting effect. Although the growth of the animals on diet XII was rather low in comparison to the control growth in exp. 6, no satisfactory explanation can be given for the difference between diets XI and XII. Anyhow, this difference hampers the quantitative interpretation of the results of this feeding trial. Comparison to the growth on the diets I, II, III and IV and the control diets shows that in this experiment fraction 2 contained nearly all the inhibiting substance, whereas fraction 3 was almost entirely harmless.

The growth on the reconstituted rye oil (diet IV) was somewhat better than on diet I. This difference however was not significant (2 P = 0,08). When comparing these results with those of the petrol fractionation carried out in experiment nr. 8 it can be concluded that the results of both experiments agree well with each other.

In comparison with the growth on diet X (1,5% fraction 2) the growth of the rats on the diets V, VI and VII in exp. 9 shows a striking difference with the growth on the corresponding diets in exp. 8. In exp. 9 the growth inhibiting effect was divided over the fractions 6 and 7, whereas in exp. 8 the total inhibiting effect was localized in fraction 7. Thus it is evident that cooling of the methanolic solutions to temperatures below + 2° C leads to the precipitation of a part of the growth inhibiting material.

Summarizing the results of the solvent fractionations it can be concluded that the major part of the rye factor appears in the fractions petrol soluble (2) and soluble in methanol at + 2° C (exp. 8, fraction 7) and that in exp. 9 the fractions 6 and 7 contained approximately the same amount of inhibiting substance. The growth inhibiting effect of the small fraction 3 could not be established with certainty whereas most probably fraction 5 (exp. 8 and 9) and fraction 6 (exp. 8) were devoid of any growth inhibiting substance.

CHAPTER V

THE ISOLATION OF THE RYE OIL FACTOR

1. Examination of rye oil fractions by means of thin layer chromatography

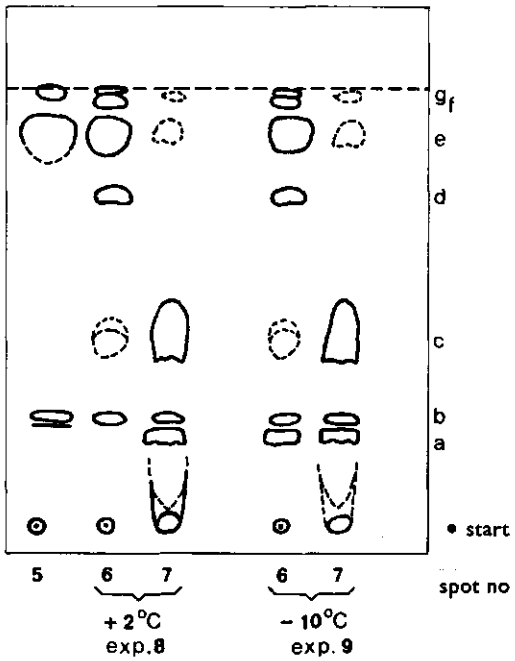
To get a better understanding in the chemical composition of the fractions obtained by the solvent fractionation as described in chapter IV the rye oil fractions were separated by means of thin layer chromatography (TLC). Thin layers of silicagel were prepared with a Desaga TLC applicator on 20 x 20 cm glass plates. A slurry of 25 g Silica G (Merck) and 60 ml water was divided over 5 plates and after air drying the plates were activated at 120° C for 1 hr and then stored in an exsiccator over silicagel. From the abundance of eluents summarized by STAHL (1962) a number were tested. In first instance the light petrol-ethyl-ether-acetic acid mixtures 90:10:1 and 80:20:2 gave satisfactory results. The latter solvent mixture was preferred for the examination of the rye oil fractions because it gave a better separation of a number of substances showing low r.f. values with the first mentioned eluent.

To indicate the spots a number of colourreagents like iodine vapour, Dragendorf reagent, phosphormolybdenic acid, $FeCl_3$, Antimone-III-chloride and vanilin phosphoric acid were tried as well as U.V. examination and charring with concentrated sulphuric acid (see STAHL 1962). On thin layer chromatograms of rye oil developed with light petrol-ethylether-acetic acid (80:20:2) vanilin phosphoric acid gave a greater number of spots (of a better sharpness) than the other reagents. After spraying with vanilin phosphoric acid the plates were heated at 120° C for 20 min. to indicate the spots.

Results of the TLC of the rye oil fractions obtained in the exp. 8 and 9 (chapter IV) are shown in fig. 5 which represents a line drawing of a developed and coloured thin layer plate. At the start a greyish spot of phospholipids appeared. The spot numbered *a* was of a very bright red colour which however discoloured to indeterminately brown within an hour. The spot *b* which sometimes faintly appeared as a double spot was coloured blue and probably belongs to the sterol fraction. The spots *c*, *d* and *e* were all more or less blue coloured and are, according to r.f. values given by STAHL mono- di-, and triglycerides; *g* and *f* also coloured blue, probably are sterol esters.

In comparing the chromatograms of the fractions 5, 6 and 7 of exp. 8, three important differences are to be noted between the harmless fractions 5 and 6 and the growth inhibiting fraction 7: fraction 7 shows much more material at the start than the other fractions; the red coloured spot *a* does not occur in the fractions 5 and 6, and fraction 7 contains considerably more of substance *c*. Looking to the difference in chemical composition between the fractions 6 and 7 of exp. 8 and those of exp. 9 it is clear that the only striking difference shown, is the partial migration of substance *a* to fraction 6 (exp. 9). This corresponds perfectly with the fact that the fractions 6 (exp. 9) and 7 (exp. 8 and 9) inhibited

FIG. 5. (Exps. 8 and 9) Line drawing of a thin layer chromatogram of rye oil fractions.



the growth of rats, whereas the fractions 5 (exp. 8 and 9) and 6 (exp. 8) appeared to be harmless. Thus the substance *a* (fig. 5) occurring as a red spot on the vanilin phosphoric acid treated chromatograms could be pointed out as the probable growth inhibiting factor.

TABLE 18. Occurrence of substance *a* in the rye oil fractions obtained in the experiments 7, 8 and 9

experiment	rye oil fraction		nr.	substance a
7	fatty acids			+
	unsaponifiable			++
8	light petrol			
		soluble	(2)	+
		insoluble	(3)	++
	methanol			
		insoluble ± 60° C	(5)	—
		precipitate + 2° C	(6)	—
		soluble + 2° C	(7)	++
9	light petrol			
		soluble	(2)	+
		insoluble	(3)	+
	methanol			
		insoluble ± 60° C	(5)	—
		precipitate — 10° C	(6)	+
		soluble — 10° C	(7)	+

In connection with this, it was of interest to examine rye oil fractions described in chapter IV by means of TLC. 10 microl. of a 1% solution of these fractions in acetone were spotted on the silica plates and after developing and spraying, the intensity of the spots was noted with + or ++ in table 18. From these figures it can be seen that in exp. 7 the fraction 'unsaponifiable' contained more of the substance *a* than the fatty acids fraction. This is in good accord with the conclusion previously drawn, that unsaponifiable matter of rye oil presumably contained more of the growth inhibiting substance than an aliquot part rye oil and that the fatty acids were relatively less harmful. In the same way the very small fractions 3 (exp. 8 and 9) were relatively rich in substance *a*. In both experiments fraction nr. 5 was totally devoid of this substance.

Thus the results of the TLC test were in all instances in accord with the results of the feeding trials and this supported the view that substance *a* was responsible for the growth inhibiting effect of rye oil.

2. The isolation of substance *A*

In order to prove the growth inhibiting action of substance *a*, this substance had to be prepared on a large scale. For this reason a column chromatographic separation method was developed. In analogy of the TLC separation silicagel columns were used. In the first experiments Malinckrodt silicagel was used. Lateron Ketjensil 101 showed a somewhat better separation, and was used for the further column chromatographic separations.

The Ketjensil 101[1]) was sieved on a AZO S_3 continuous sieving apparatus[2]) over a 60 μ sieve and the material $> 60\ \mu$ was dried and activated overnight at 120° C. 200 g of this silicagel was mixed with 1600 ml of a mixture of light petrol-ethylether-acetic acid (80:20:2) and poured in a 6 cm wide chromatography tube. After settling and drainage of the superfluous eluent a column of 38–40 cm was obtained. Above the top of the column a free space of 15 cm was left. 10 grams of the rye oil fraction 7 (exp. 8) were carefully brought onto the toplayer of the column and directly after absorbtion of the oil in the silicagel the column was eluted with light petrol-ethylether-acetic acid (80:20:1). Fractions of 100 ml were collected, and after having collected 17 fractions a further elution was carried out with methanol in order to regain residual material from the silicagel. TLC examination of the fractions showed that the separation in this type of column was disappointingly incomplete (table 19).

Not only could the substance *a* not be separated from *b*, but in the fractions nrs P_2 and P_3 substance *c* overlapped *a* and *b*. Other solvent ratios of the eluent could not enhance the results with regard to the separation of *a* and *b*. Another possible way to reach a better separation was the lengthening of the column. The disadvantage of the lower elution velocity however made the application of longer columns impracticable.

[1]) Ketjensil 101 (Ned. Verkoopkantoor Chemische produkten Amsterdam).

[2]) The sieving was conducted by the Central Technical Institute TNO Delft. The statement that the material $> 60\ \mu$ was used does not implicate that the particles were $> 60\ \mu$. In fact by sieving, only a separation of differently sized clusters of particles was achieved.

TABLE 19. Exp. 10: Results of the column chromatographic separation of rye oil fraction 7 (exp. 8) petrol-ethylether-acetic acid elution

fraction nr.	code	component
1– 7	–	–
8–11	P_1	c, d, e, f, g*
12–13	P_2	a, b, c
14–20	P_3	a, b
21–30	P_4	phospholipids

* for explanation of the letters see fig. 5.

In order to find a better method to separate the substance *a* and *b* a number of eluents was tested on thin layer chromatograms. It appeared that chloroform-acetone 90:10 or 85:15 resulted in higher r.f. values for *a* and *b* so that these compounds were not separated from *c* and *d*. Thus it was thought that it might be possible to isolate substance *a* on columns after a preceding isolation of substance *a* and *b* together.

To obtain a good separation on silicagel columns, the ratio chloroform-acetone had to be altered to 97,5:2,5. The chromatography tube was filled with a slurry of 250 g sieved and activated Ketjensil 101 with 1600 ml chloroform-acetone (97,5:2,5). After settling of the silicagel and drainage of the superfluous eluent, 2 grams of fraction P_3 (freed from the light petrol eluent) was brought on the top of the column. After adsorbtion in the top layer the column was eluted with 2000 ml of chloroform-acetone followed by 600 ml methanol. The fractions of 100 ml were tested on thin layer plates eluted with chloroform-acetone (85:15). It was shown that substance *b* was composed of at least 3 components which appeared in the fractions 8 and 9 (see table 20).

TABLE 20. Exp. nr. 10: Results of the separation of substance *a* and *b* on columns eluted with chloroform-acetone (97,5:2,5)

fraction		component
nr.	code	
1–7	–	–
8–9	C_1	substance *b* (3 components)
11–17	C_2	substance *a*
18–23	C_3	substance (*a*) + *x*
23–28	C_4	residual material

Substance *a* appeared in the fractions 11–17. On the thin layer chromatograms no other substances could be detected in these fractions. Due to tailing in the column minute amounts of *a* were present in the fractions 18–23. Beside these, in these fractions another substance x occurred which appeared on the thin layer plates as a greyish blue spot with a slightly lower r.f. value than substance *a*. The residual material, washed out by the methanol, was collected

in the fractions 23–28. 300 g of the rye oil fraction 7 (exp. nr. 8) was chromatographed according to the 2 column method described. Fraction C_2 (table 20) and the combined fractions P_1, P_2, P_4, C_1, C_3 and C_4 (see table 19 and 20) were freed of solvents in the rotating vacuum evaporator. Special care had to be given to remove the acetic acid. Hence in the final phase of destillation small amounts of acetone were added in the evaporator to enhance the evaporation of evaporation of acetic acid residues. The fractions obtained in this way from the rye oil fraction 7 can be defined as 'substance *a*' and 'fraction 7 – *a*'. In total 24 g of substance *a* and 283 g 'fraction 7–a' were produced from the 300 g of fraction 7 (exp. 8). As can be concluded from the tables 19 and 20 the fractions P_2 and C_4 were contaminated with substance *a*. At a rough estimate this contamination remained below 5 % of the total amount of substance *a*.

Substance *a* and 'fraction 7–*a*' were tested in a feeding experiment with rats. In the same way as in the foregoing experiments, maize oil was replaced in the basic diet (table 9) by the fractions under investigation broadly in proportion to the percentages in the composition of rye oil fraction 7 (exp. 8). In analogy to exp. 8 and 9 fraction 7 was reconstituted from its components and also tested. The amounts of the fractions added to the diets and the figures on consumption and growth of rats fed on these diets are given in table 21.

TABLE 21. Exp. nr. 10: Average growth of rats on diets with substance *a* during 2 weeks

diet	rye oil		substance *a* in % of the diet	surviving animals	food-consumption g	growth g
	fraction	in % of the diet				
I	–	–	0,00	8	134	43
II	7	3	0,25	4	82	16
III	7–*a*	2,7	0,01[1])	10	135	37
IV	*a*	0,3	0,30	3	89	10
V	7–*a*	2,7	0,31	1	65	–1
	a	0,3				

[1]) estimated

Due to the fact that the percentage of substance *a* added to diet IV was rounded off upwards and that the fraction '7–*a*' was contaminated with appr. 5 % of substance *a*, the diets II, IV and V were not quite comparable with regard to the substance *a* content. Although in planning the feeding trial these differences were considered to be of minor importance, a noticeable effect of these differences could be observed during the experiment. Obviously, the batch of rye-grit and -bran used for the extraction and further separation procedures contained more growth inhibiting substance than the rye-grit and -bran used in the previous experiments. Although a diet with 3 % fraction 7 (exp. 8) approximately equalled a diet with 5 % rye oil, in this experiment 6 of the 10 animals on diet II died within the 2 weeks of the feeding trial. Mortality on the diets IV and

V was even greater. The differences in deathrate were however not significant. No explanation can be given for the death of 2 animals of the control group.

Comparing the growth results of the surviving animals, a remarkable difference can be observed between the diets I and III on the one hand and the diets II, IV and V on the other hand. The animals on the latter diets containing 0,25, 0,30 and 0,31 % of substance *a* respectively grew significantly less than the animals on the control diet and diet III with approximately 0,01 % substance *a*. Due to the high mortality in the groups of rats receiving substance *a* no levels of significance can be calculated. Undoubtedly, however, it can be concluded from this experiment that substance *a* is the only agent responsible for the growth inhibiting effect of rye oil.

3. Note on the effect of the basic diet on the health of rats

Post mortem examination of the rats used in exp. 10 showed abnormal organs in all animals. The majority of the rats which received rye oil or substance *a* appeared to have pale kidneys, with dark spots. In a number of cases such haemorrhages were also observed in the liver and the lungs and in one case haemorrhages of the heart muscle was observed. The animals fed on the control diet or on rye oil, freed from substance *a* did not show haemorrhagic organs, but in these animals distinctly anaemic kidneys were observed, and in some animals pale livers and lungs as well.

Without neglecting the haemorrhages, observed in the organs of the experimental animals, the primary cause of the abnormal paleness of the organs of the control animals was sought in the basic diet. To find out whether the anomalies were caused by the maize oil or by the other constituents of the basic diet, 4 feeding trials, with a total of 126 female and 40 male rats were carried out. In these experiments the following diets were used: the basic diet (table 9) with maize oil or with peanut oil instead of maize oil and the C.G. diet described in table 29 with peanut oil or with maize oil.

The main results of these experiments which will not be described in detail here, are the following:

1. Pale (or anaemic) organs occurred in only a part of the animals fed on the old basic diet with maize oil.
2. Substitution of peanut oil for maize oil in the old basic diet resulted in normal organs.
3. Replacement of a part of the peanut oil in the old diet by rye oil resulted in normal kidneys and other organs in one experiment and some cases of haemorrhagic kidneys in another experiment. In both experiments the rye oil caused a growth depression however.
4. Animals receiving the C.G. diet with maize oil, peanut oil or peanut – and rye oil did not show abnormal organs.
5. Apparently the abnormal organs occurred in the animals irrespective of age and sex.

Although no explanation can be given for the observed anomalies it was concluded that the old basic diet with maize oil under certain conditions may result in undesirable side-effects. For this reason the C.G. diet with peanut oil was used for the further experiments.

Irrespective of the kind of aberrance or deficiency in the old basic diet, it can be stated that the effect of rye oil superimposed on that of a certain insufficiency in a diet may result in haemorrhages of the organs.

CHAPTER VI

EXPERIMENTS ON THE CHEMICAL NATURE OF SUBSTANCE *A*

1. THE IDENTIFICATION OF SUBSTANCE *A*

To elucidate the structure of the growth inhibiting factor, substance *a* was submitted to a mass spectrometric analysis[1]). The first spectrum showed, in sequence of decreasing peak heights, peaks at the mass numbers 348, 376, 320, 404, and 432. These were accompanied by smaller peaks with mass numbers 1 or 2 units less than the main peaks. This indicated that substance *a* was not a pure compound, but a mixture of 5 components, accompanied by small amounts of presumably corresponding mono- and di-unsaturated homologs. Secondary peaks caused by the breakdown products were shown at mass numbers 166 and very markedly at 124. Because the IR spectrum[1]), which indicated the occurrence of hydroxy groups and of an aromatic ring, showed a certain resemblance to the IR spectrum of orcinol (3,5 dihydroxytoluene (MW 124), it was presumed that substance *a* might be a mixture of homologs of orcinol.

Further mass spectrometric examination showed that substance *a* was contaminated with dibutyl – and dioctylphtalate which probably were introduced in substance *a* by the organic solvents. After a prolonged stay of the sample in the mass spectrometer the influence of the contaminating substances decreased. In the same time however the peak at mass number 376 became predominant over the 348 peak. No correlation could be detected between this shift in peak heights and the peaks at 166 and 124. This supported the hypothesis that substance *a* was a mixture of homologous compounds, differing C_2H_4 from each other, and all producing breakdown products with mass numbers 116 and 124.

Recently OCCOLOWITZ (1964) found that 5-pentadecyl resorcinol (MW 320) and related compounds also showed ion peaks at 166 and 124. This confirmed the opinion that substance *a* was a mixture of resorcinol derivatives with side chains of 15, 17, 19, 21 and 23 C atoms respectively.

For further purification, substance *a* was dissolved in warm methanol and recrystallized at –10° C. The precipitate was distilled at 170° C and 10^{-2} Torr in a cold finger sublimator. A white powder was obtained with a melting point of 83° C (substance *a* m.p. 67°–68° C). In the sublimator a dark brown oily residue was left, which indicated thermal breakdown of at least a part of the material. Hence another sample of substance *a*, after recrystallization was distilled at 140° C (10^{-2} Torr). Again a white powder was obtained (m.p. 82° C) but the yield was smaller, whereas the residue was only slightly darkened. At mass spectrometric examination, no contamination with phtalates could be detected. The spectrum of the purified preparations differed from that of the crude sub-

[1]) Thanks are due to Dr. C. Engel, director of the Central Institute for Nutrition and Food Research TNO, Zeist, and his collaborators Dr. R. J. C. Kleipool and Dr. C. D. Leegwater for mass spectrometric, IR and gaschromatographic analysis.

stance *a* in so far that the peak at mass number 432 was not observed in preparation 2 (140° C).

The purified preparations were submitted to elemental analysis[1]). For the preparations the following composition was found:

preparation 1: 80.12% C; 11.75% H and 8.35% O
preparation 2: 79.99% C; 11.78% H and 8.52% O[2])

Presuming 2 atoms of O per molecule, the elementary composition of these preparations can be calculated as $C_{25.7}.H_{45.2}.O_{2.0}$ and $C_{25,1}.H_{43,7}.O_{2,0}$ or rounded $C_{26}.H_{46}.O_2$ (MW 390) and $C_{25}.H_{44}.O_2$ (MW 376).

These results are in accord with the results obtained with IR and mass-spectrometric analysis. According to the elemental analyses the average chain length of the preparations 2 and 1 comes to 19–20 C atoms. Because the mass spectrum did not allow conclusions on the quantitative composition of the mixture, the preparations 1 and 2 were acetylated and separated gaschromatographically[3]). Five and four peaks resp. were obtained and interpreted according to the mass spectrum belonging to the compounds with the molecular weights 320, 348, 376, 404 and (432) respectively. The composition of each of the two preparations, calculated in percentages of total peak area is given in table 22.

TABLE 22. Composition of the preparations 1 and 2

compound with molecular weight	chain length in C atoms	relative amount in % of total peak area	
		preparation 1	preparation 2
320	15	1	5
348	17	11	35
376	19	44	52
404	21	41	8
432	23	3	–
average chain length	in C atoms	19.7	18.3

From these figures it can be concluded that a higher destillation temperature (prep. 1) resulted in considerably higher amounts of the compounds with a higher molecular weight than the lower destillation temperature (prep. 2). The average chain length of the preparations calculated from GLC data perfectly agrees with the chain length based on the elemental analysis.

To locate the position of the hydroxy groups, a number of qualitative reactions was carried out according to the methods described by KARRER (1947) and BUTENANDT and STODOLA (1939). Phosphormolybdenic acid, when added to a solution of substance *a* in glacial acetic acid did not give a colour reaction. Subsequent addition of ammonia, however, caused a blue colour, which pointed

[1]) Thanks are due to Mr. W. P. Combé, Laboratory for Organic Chemistry of the Agricultural University Wageningen, for carrying out the elemental analyses.

[2]) Calculated.

[3]) See note 1, page 41

to a 1. 3-dihydroxy benzene structure. Alcoholic solutions of substance *a* did not give a colour reaction with ferric chloride, nor with silver nitrate; mercuric nitrate however caused an abundant white precipitate. By these results the 1.2 – and 1.4 – dihydroxy benzene structures could be excluded and in all probability the hydroxy groups in substance *a* occur in the 1.3 position.

BUTENANDT and STODOLA (1939) described a number of colour reactions to assess the number and the position of the side chains in resorcinol derivatives. Their investigations included compounds with side chains at the position 4; 5; 4 and 6; and 4, 5 and 6. Of these compounds only 5-alkyl resorcinols gave a negative test with ferric chloride, like substance *a*. For further evidence, substance *a* was dissolved in glacial acetic acid and after addition of sulfuric acid and a few cristals of sodium nitrite, no colour reaction occurred (Liebermann-indophenol test). A positive reaction would have pointed to a side chain on the 4 position. Heating of substance *a* with chloroform and KOH gave a bright red colour (Guareschi-test). 4- and 5-alkyl resorcinols gave a positive Guareschi-test, whereas 4.6-dialkyl- and 4.5.6-trialkyl resorcinols are negative. From the results of these three colour reactions it seems warranted to conclude that substance *a* can be defined as a mixture of 5-alkyl resorcinols.

2. DISCUSSION ON THE OCCURRENCE OF HITHERTO KNOWN DIHYDROXY ALKYLBENZENES IN NATURE

A superficial survey of the literature showed already that alkyl derivatives of dihydroxy benzene are very common in the family of the Anacardiaceae. HAACK (1940) listed a number of species belonging to this family which were known to contain catechol – or resorcinol derivatives in the bleeding sap, the leaves, or the fruits. Among those species are Rhus toxicodendron, Semecarpus species, Gluta renghas, and Melanorrhoea species, from which catechol derivatives like urushiol, renghol, laccol, glutarenghol, and thitsiol can be isolated. The side chain in these compounds amounts to 15 or 17 C atoms and is situated ortho or meta to one of the hydroxy groups. All these compounds have in common one or more double bonds in the side chain, and the property of causing skin irritation or even painful blisters and inflammations.

Of more interest, in connection with the resorcinol derivatives occurring in rye, are the products cardol, and bilobol, which occur in the fruits of Anacardium occidentale Linn. and Ginkgo biloba (a gymnosperm). These substances respectively are di- and mono unsaturated resorcinol derivatives with a 15 carbon side chain in meta position to the hydroxy groups. Both substances possess vescicant properties and, under certain conditions, may become alergenic. According to HAACK cardol and anacardol (3 pentadecenyl 1 hydroxy benzene) are less vescicant than renghol and other unsaturated catechol derivatives. After hydrogenation however, the catechols as well as the resorcinols fail to show any skin reaction.

CROWDER *et al* (1936) isolated dihydroxy benzenes from the acetone soluble oil of Mycobacterium leprae, which substance could be identified as a

4.5.6.-trialkyl resorcinol by BUTENANDT and STODOLA (1939). No mention is made however, on the biological activity of this compound.

Recently WENCKERT *et al* (1964)[4]) found 5–n–alkyl resorcinols in the non saponifiable fraction of wheat bran. By comparing the nmr spectrum with the spectra of the six dimetoxy toluenes the 5–alkyl resorcinol structure could be proved. Although the nmr spectrum pointed to a resorcinol with a side chain of 20 C atoms, gaschromatographic analysis showed that the substance in fact was composed of 5 compounds with straight saturated chains of 17, 19, 21, 23 and 25 C atoms. Final evidence on the structure of these resorcinols was obtained after synthesis of 5–n–heneicosyl resorcinol and 5–n–nonadecyl resorcinol.

Comparing the work of WENCKERT *et al* and the present investigation it is obvious that the closely related grain species wheat and rye are characterized by the occurrence of 5–alkyl resorcinols. Assuming that both the grain species contain 5–alkyl resorcinols, the question arises why rye has a bad reputation as a feedstuff while wheat has not. In connection herewith, it may be mentioned that CRAMPTON (1939) (see also chapter I) had already concluded that wheat, although an excellent feed for pigs, should not form more than 50% of the ration. This indicates that wheat bran resorcinols may play a part in animal feeding, but that the effect does not come to the fore possibly on account of the lower concentration of these resorcinols in wheat. Alternative possibilities are that wheat – and rye resorcinols are a) not identical at all, or b) that 5–n–alkyl resorcinols are not toxic. In these cases the growth inhibiting effect of the rye resorcinols would be due to branching or unsaturation of the side chain. Furthermore, it is not clear up till now whether the length of the side chain influences the growth inhibiting effect or not.

For this reason it was considered necessary to obtain further evidence on the chemical structure of the rye resorcinols. It should be mentioned here, that wheat is generally used for human consumption, whereas rye – in Western Europe – has always had an important part in animal feeds. Farmers on sandy soils, on account of the difficult economic circumstances, wished to utilise their own rye as much as possible in animal feeds. Possible difficulties might therefore be expected in the first instance with rye.

3. FURTHER EXPERIMENTS ON THE CHEMICAL STRUCTURE OF RYE RESORCINOLS

To find out whether the rye resorcinols were identical to the resorcinols isolated from wheat bran, wheat resorcinols were included in the further experiments, and rye resorcinols were tested according to some of the methods described by WENCKERT *et al* (1964).

Firstly an acetone extract of wheat was tested according to the method described in chapter V. 1. On thin layer chromatograms wheat oil showed a red spot with the same r.f. value as that of substance *a*. Compared to rye however the red spot was smaller and less clear.

[1]) Thanks are due to Dr. N. H. HAACK who focussed my attention on this work.

Nuclear magnetic resonance (nmr) analysis[1]) of preparation 2 (chapter VII.1) was carried out on the JNM C 60 (Jeol, Japan), in deuterioacetone with tetramethylsilane (t = 10 ppm) as an internal standard. Apart from a slight chemical shift of the absorptions to a higher field, the results were in correct accord with those obtained by WENCKERT and coworkers. The nmr spectrum showed a 3-proton broad singlet at 9,25 ppm (C–CH_3), a 33–36 proton broad singlet at 8,85 ppm (16–18 CH_2), a 2 proton multiplet at 7,65–8,05 ppm (benzylic protons), a 3 proton broad singlet at 4,00 ppm (aromatic protons) and 2 singlets of in total 2,5 protons at 7,05 and 2,00 ppm (hydroxylic protons). From these results it can be concluded that the occurrence of branched side chains in the rye resorcinols is highly improbable.

Wheat and rye resorcinols were prepared chromatographically as described in chapter V and pentadecyl resorcinol (Noury–Baker) was purified in the same way, recrystallized twice from methanol, and distilled at 140° C, 10^{-2} Torr. The ultraviolet spectrum of these three preparations showed λ max. at 275 and 281 nm.

The crude rye resorcinol preparation and the recrystallized and distilled pentadecyl resorcinol were methylated with dimethylsulfate in a mixture of dry acetone and anhydrous potassium carbonate, as described by WENCKERT *et al.* Gas phase chromatography[2]) of the dimethyl ethers, according to WENCKERT *et al* showed that the pentadecyl resorcinol dimethylether was a single substance whereas the rye resorcinol dimethylether preparation was composed of 6 compounds, one of these being identical to pentadecyl resorcinol dimethylether. The composition of the mixture is given in table 23.

In comparison to the composition of the purified preparations 1 and 2 (given in table 22) the crude preparation even contains resorcinols with 25 C side chains. It will be clear that during purification some compounds of the mixture are eliminated. Hence the composition of the crude preparation will give a more

TABLE 23. Composition of crude resorcinol mixtures from rye and wheat (in % of total peak area)

compound with chain length in C atoms	resorcinols from			
	rye		wheat	
	methylated	acetylated	methylated	acetylated
15	2	2	–	1
17	27	40	4	8
19	37	43	34	48
21	24	13	48	42
23	7	1	9	1
25	3	–	5	trace
average chain length	19,3	18,4	20,5	19,7

[1]) The author is indebted to Prof. Dr. H. J. DEN HERTOG, Dr. H. C. VAN DER PLAS and Drs. P. SMIT, Laboratory for organic Chemistry of the Agricultural University, Wageningen, for invaluable advice and the n.m.r. analysis.

[2]) See note 1, page 41.

accurate picture of the actual composition of resorcinols in rye than the purified preparation.

In comparison to the composition of a methylated rye resorcinol mixture the composition of acetylated rye resorcinols (obtained from another sample of rye) showed a shift to a lower average chain length (table 23). It cannot be concluded with certainty that this is due to a natural variation in resorcinol chain length in rye because this difference may also be caused by the method of preparation, incomplete acetylation or even a certain retention of the acetylated long chain compounds in the gaschromatograph.

The composition of the acetylated wheat resorcinols in comparison to the acetylated rye resorcinols shows a longer average chain length, in comparison to the composition as found by WENCKERT *et al*, however a shorter average chain length (table 23).

Wheat and rye resorcinols, prepared chromatographically, were hydrogenated[1]) in aethanol under atmospheric hydrogen pressure, at room temperature with 10% Paladium on charcoal as the catalyst. Under these conditions, pentadecyl resorcinol did not absorb hydrogen whereas the grain resorcinols consumed:

0,43 and 0,45 m.mol. H_2/g rye resorcinols

0,48 and 0,50 m.mol. H_2/g wheat resorcinols.

Accepting an average molecular weight of 370 for both the preparations and presuming that the crude preparations did not contain unsaturated impurities, 16,9 and 18,8% alkenyl resorcinols can be calculated to occur in the rye – and wheat preparations respectively. These figures are much higher than was expected from the mass spectrometric data, indicating the occurrence of appr. 1% of alkenyl resorcinols in the purified (rye) substance *a*. Furthermore, as much as 15% of alkenyl resorcinols in the mixture inevitably would have been detected in the n.m.r. peak diagram, which was not the case, however. The antithesis between these results is probably due to the fact that during recrystallization and distillation unsaturated compounds are eliminated from the crude resorcinol preparations. It cannot be concluded however, if, and in how far the unsaturated compounds are alkenyl resorcinols or other unsaturated (low molecular) compounds, occurring as impurities in the crude preparations.

[1]) Thanks are due to Mr. W. P. Combé, Laboratory for Organic Chemistry of the Agricultural University Wageningen, for the hydrogenation of te samples.

CHAPTER VII

THE CHEMICAL DETERMINATION OF 5-ALKYL RESORCINOLS

1. THE SEMIQUANTITATIVE DETERMINATION OF 5-ALKYL RESORCINOLS

For screening a great number of rye varieties a useful method of analysis has to meet a number of requirements. Because great numbers of small or extremely small samples are involved in breeding work, the analysis of the substance under investigation has to be as sensitive and as simple as possible.

The TLC method described in chapter V.1 conforms to the requirements of sensitivity and relative simplicity. The qualitative character of TLC methods however is a drawback, which can only be partly overcome by roughly estimating size and intensity of the spots in comparison to known amounts of a suitable reference sample. To test the sensitivity of the TLC method, the purified pentadecyl resorcinol (see chapter V and VI) was used. 0,25 μ g pentadecyl resorcinol could be detected (fig. 6). Comparison of chromatograms of rye extracts with the pentadecyl resorcinol scale (fig. 6) showed that rye contains 0,05–0,1 % alkyl resorcinols, which is in correct accord with the 0,08 % calculated from the column chromatographic separation described in chapter V. 2. For the qualitative demonstration of resorcinols in rye, an acetone extract of rye representing 1 mg of rye is sufficient to cause a clearly visible spot on the thin layer plate. Thus it can be concluded that for the qualitative demonstration of resorcinols one rye kernel or even a part of one kernel is sufficient. It will be clear however that only great differences in resorcinol content can be estimated in this way.

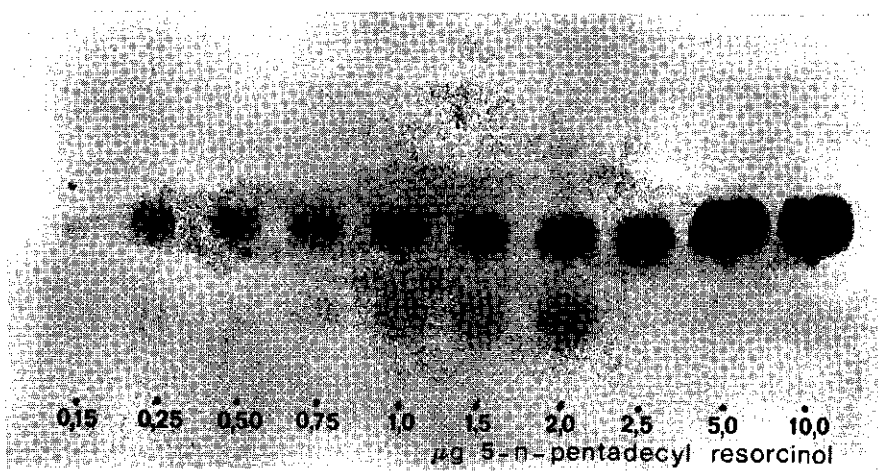


FIG. 6. Thin layer chromatogram of different amounts of 5-n-pentadecyl resorcinol.

2. The quantitative determination of 5-alkyl resorcinols

In order to develop a method for quantitative analysis, a sensitive and specific reaction is needed. The Guareschi test, described in chapter VI.1. can be considered as one of the most sensitive and specific reactions on 5-alkyl resorcinols. For this reason it was attempted to modify this qualitative reaction to a reproduceable quantitative determination.

One of the first difficulties met, was that the red colour developed by heating rye resorcinols with chloroform and KOH did not change into a green fluorescence after adding water to the reaction mixture (Haack 1940). Considering that this might be due to the fact that the long chain alkyl resorcinols are insoluble in water, diluted aethanol was used instead of water. It was shown that by the addition of 50 ml 75–87 % aethanol to 2 ml of the chloroform alkyl resorcinol-KOH reaction mixture a green fluorescence could be obtained. Aethanol between 75 and 87 % did not influence the fluorescence.

Although on the ground of this result it was decided, to develop the Guareschi test to a quantitative fluorimetric method, it appeared that the red colour produced by heating alkyl resorcinols with chloroform and KOH was heat- and alkali-labile. In the incubator at 70° C the red colour grew faint within a few minutes and then turned yellow, resulting in a considerable decrease of fluorescence after adding water and aethanol. Lower temperatures, especially below the boiling point of chloroform led to a slowing down of the reaction and non-reproduceable results. This was probably also due to the fact that in this case the stirring effect of boiling chloroform did not occur. Therefore a laboratory shaker was used in further experiments. It was shown that in this way, given a suitable KOH concentration, at 35° C the reaction could be kept under control.

Powdered KOH granules (appr. 50 mg/2 ml $CHCl_3$) as used originally, after 5 min. shaking at 35° C would already cause a decreased fluorescence. Lowering the amount of KOH to 10 mg led to an incomplete reaction. Furthermore it was shown that in this case fluorescence could only be obtained after adding KOH to the water-aethanol mixture.

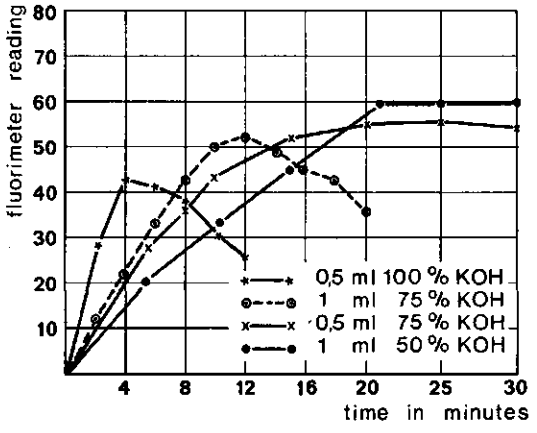


Fig. 7. Influence of KOH concentration and reaction time on fluorescence of 5-n-pentadecyl resorcinol.

To overcome the difficulty of a breakdown of the red substance by alkali at lower temperatures, a number of experiments was carried out with KOH solutions. 0,5 ml KOH 100 % (w/v)/2 ml chloroform gave the same results as 50 mg powdered KOH. 0,5 ml 75 % KOH and 1 ml 50 % KOH in time showed a gradual increase in fluorescence and maximum fluorescence was maintained during a reasonable time (fig. 7). With 1–2 ml 25 % KOH no reaction at all was observed.

Based on the above mentioned results the determination of 5–alkyl resorcinols was carried out as follows:

Reagents

- Pentadecyl resorcinol (Nouri-Baker) purified column-chromatographically and distilled at 140° C 10^{-2} Torr (chapter V)
- Chloroform, reagent grade
- KOH, reagent grade
- Aethanol 96 % (or denaturated with 10 % methanol)
- Distilled water or deionized water.

Procedure

- Solutions of purified pentadecyl resorcinol in chloroform, containing 0, 5, 10, 15, 20 and 25 µg/ml are prepared
- Acetone extracted rye oil samples are dissolved in chloroform in such a way that 1 ml of the solution represents appr. 20 mg rye. (Rye may be extracted with chloroform, but this takes longer extraction time than acetone extraction.)
- 2 ml of these solutions are pipetted in 50 ml volumetric flasks and 1 ml 50 % KOH (w/v) is added per flask. The flasks are shaken for 25 min. at 35° C on a laboratory shaker (appr. 500 (double) strokes/min.; stroke length 1–2 cm)

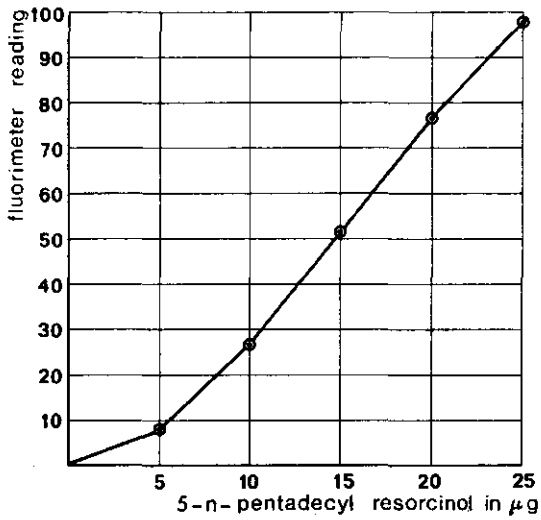


FIG. 8. Standard line.

- Immediately after this, the reaction is stopped by adding 5–7 ml water, and under gentle shaking the flasks are filled to the mark with aethanol
- After 30 min. at room temperature, maximum fluorescence is reached and can be measured.

Apparatus

Fluorescence was measured with a Vitatron fluorimeter (Vitatron N.V. Dieren, the Netherlands) with a Hg lamp as the light source, a U 8 primary filter (350–500 nm) and a U 12 secondary filter. 1 cm optical cuvettes were used.

The zero and 10 μ g pentadecyl resorcinol are used for the 0 and 100 setting respectively. A standard line is made by measuring the other pentadecyl resorcinol concentrations. Fluorimeter readings of the experimental samples can be read as μ g pentadecyl resorcinol from the standard line (fig. 8). Because small differences in fluorimeter readings may occur from day to day the standard line has to be run with each series of determinations.

Calculation

When the acetone extract of *a* mg of rye, after the evaporation of the acetone, is dissolved in *v* ml chloroform and the fluorimeter reading corresponds to s μg pentadecyl resorcinol, the amount of 5-alkyl resorcinols (calculated as pentadecyl resorcinol) in rye is:

$$\frac{100\ v}{a} \times s \text{ mg } \% \ (= \text{mg}/100 \text{ g}).$$

CHAPTER VIII

LOCALIZATION OF RESORCINOLS IN THE RYE KERNEL

1. THE GROWTH INHIBITORY EFFECT OF RYE MILLING FRACTIONS

As mentioned already in chapter I DAMMERS (1955) concluded that the growth inhibitory substance occurred for the greater part in the bran and grit and that rye flour is less harmful than rye grit and bran. Because the rations used, contained large percentages of rye or barley milling fractions, the comparability of the ration (as to the energy and protein value) could not be assessed with certainty. The finding, that the growth inhibitory substance was extractable with acetone (chapter III), created the possibility to estimate the differences in harmfulness between flour, grit and bran by means of a feeding trial in which the acetone extracts of the milling fractions were tested.

For experiment nr. 11 1000 kgs of 'Petkuser' winter rye were milled on a rye flour mill of Koopmans Meelfabrieken, Leeuwarden, and the milling fractions obtained were extracted with acetone in a stainless steel batch extractor by the Central Technical Institute, Delft. Some data on the milling and the acetone extraction are given in table 24.

TABLE 24. Exp. nr. 11: Distribution of acetone soluble oil over the milling fractions of rye

milling fraction	acetone soluble oil			
	weight in kg	in % of the fraction	in g	in % of total rye oil
flour (export grade)	650	1,0	6500	35
flour (feed grade)	30	2,2	660	4
grit	310	3,7	11470	61
bran	1	3,8	38	–
rye (total)	991	1,9	18668	100

From the results it can be seen that at the extraction rate of 65%, applied in the mill, 65% of the total acetone soluble material from the rye is found again in the feed grade millery products and 35% in the flour (export grade). The oil fractions obtained from rye, flour (Eg) flour (Fg) and grit were tested in a feeding trial with young rats of appr. 40 g weight according to the method described in chapter III. 5% maize oil in the basic diet (table 9) was replaced by 5% of the oils under investigation. The results of the feeding trial which lasted 5 weeks, are given in table 25.

Comparison of the results with those of exp. nr. 6 (fig. 1) shows that the growth of the animals on 5% rye oil in this experiment is somewhat better than in exp. nr. 6, and that all the animals survived. Although the difference is small, the growth on the diet with 5% oil from rye flour (Eg) is even better than on the

TABLE 25. Exp. nr. 11: Average food consumption and growth of young male rats receiving 5% oil derived from different milling fractions of rye

oil source	food consumption in g	growth g	growth inhibitory effect in g in comparison with rye flour	number of surviving animals
rye	314	85	91	10
rye flour (Eg)	557	176	0	10
rye flour (Fg)	422	127	49	10
rye grit	212	34	142	9

control diet in exp. nr. 6. From this it can be concluded that the oil derived from flour (Eg) did not contain any growth inhibitory substance. Consumption and growth of the rats on the diet with 5% rye grit oil were lower than on the other diets. The oil derived from rye flour (Fg) held an intermediate position. According to the Wilcoxon test all differences in consumption and growth were highly significant ($2\,P < 0{,}01$).

The existence of a reverse linear relationship between the percentage of rye oil in the diet and the growth of rats enables a more quantitative examination of the results of exp. nr. 11. The growth inhibition caused by rye oil (176–85 = 91 g) amounts to 64% of the growth inhibition (142 g) caused by rye grit oil. Based on the data given in table 24 and 25, the inhibitory substance is localized in 35% of the total rye containing 65% of the total rye oil. The agreement between these figures demonstrates once more the rectilinear relationship between the amount of inhibitory substance in the diet and the growth depression.

Although the results of exp. nr. 11 affirm the finding of DAMMERS (1955) that most of the harmful substance is localized in the outer layers of the rye kernel, this experiment (provided an extraction rate of not higher than 65%) clearly demonstrates the absolute inocuousness of rye flour (Eg).

2. CHROMATOGRAPHIC EXAMINATION OF THE DIFFERENT PARTS OF THE RYE KERNEL

Although by milling the major part of the endosperm is concentrated in the flour (Eg) and most of the aleurone layer and connate pericarp in the fractions grit and bran, the separation is far from quantitative. Moreover, during milling the germ oil is devided over the different milling fractions. Thus from the results of exp. nr. 11 it cannot be concluded with certainty, in which part of the rye kernel the resorcinol derivatives are concentrated.

Therefore, a number of rye kernels were soaked in water during 2–3 hrs and dissected in different parts. The cuticle of the pericarp could be removed easily, as well as the germ. More difficulties were encountered in the separation of the endosperm and the wall of the caryopsis. Most of the endosperm was scraped off, in such way, that it was not contaminated with wall material. The different

parts obtained from 20 rye kernels were extracted with acetone, and the extracts were tested by TLC (see chapter V). The results in table 26 show that the cuticle, endosperm and the germ do not contain resorcinol derivatives, but that all the material causing a red colour after reaction with vanilin phosphoric acid occurs in the caryopsis wall.

TABLE 26. Occurrence of resorcinols in different parts of the rye kernel

part of the kernel	resorcinols
cuticle	–
germ	–
endosperm	–
aleurone + pericarp	+

These results confirm those of exp. nr. 11 as far as showing that the endosperm does not contain resorcinol derivatives. By TLC examination, however, the resorcinols can be localized more precisely in the caryopsiswall. Because an efficient separation into aleurone and pericarp was not possible, the suggestion, that in analogy to the occurrence of cardol in Anacardium occidentale, the resorcinols in rye probably occur in the pericarp, could not be more than a hypothesis. To test this hypothesis, sections of the rye kernel were placed on a slide, treated with vanilin-hydrochloric acid, and examined microscopically. The aleurone granules coloured pink, and the starch granules of the endosperm were hydrolyzed. Besides this it was observed, that the originally yellow-brown layer composed of the outer cuticle of the seed coat and the inner cuticle of the pericarp and which is separated from the aleurone layer by the hyaline layer became dark brown-red after treatment with vanilin-hydrochloric acid. This brown-red colouring resembling the colour of resorcinols on the vanilin phosphoric acid treated thin layer chromatograms, strongly indicated that the resorcinols in rye exclusively are localized in the two connate cuticle layers outside the hyaline and aleurone layer.

3. CONSEQUENCES OF THE UNEVEN DISTRIBUTION OF 5-ALKYL RESORCINOLS IN RYE

The finding, that the resorcinol derivatives are located exclusively in the pericarp, did anticipate that the amount of resorcinols in rye might be proportional to the surface area of the kernel and not to the weight. Because it is known, that due to environmental conditions, a great variance in kernel weight occurs, difficulties in the interpretation of differences in 5-alkyl resorcinol content of rye varieties could be expected.

To obtain a better insight in this question, resorcinols were determined in samples of rye obtained from fertilizer experiments[1]). In table 27 crude protein, 1000-kernel weight, acetone-soluble oil and alkyl resorcinols are given for three

[1]) Thanks are due to 'Rijksdienst IJsselmeerpolders' Kampen and 'Institute for Soil Fertility' Groningen for kindly providing the rye samples.

series of rye samples. Due to late nitrate fertilization, great differences in 1000–kernel weight and protein occurred in each series.

According to expectations, the heavier kernels contained relatively less resorcinols than the small ones. Because in this experiment a positive correlation occurred between 1000–kernel weight and crude protein content, it could not be concluded, if the alkyl resorcinol content was also correlated directly to the crude protein or not. No correlation was observed between alkyl resorcinols and acetone-soluble oil in these samples.

TABLE 27. Crude protein, 1000-kernel weight, acetone-soluble oil and 5–alkyl resorcinols in Petkuser rye obtained from fertilizer experiments

sample	crude protein %	1000-kernel weight[1]) g	acetone-soluble oil %	alkyl resorcinols relative values[2])
A 1	9,4	32,8	1,5	47
A 2	11,6	33,8	1,5	45
A 3	12,9	35,1	1,4	41
A 4	14,8	34,3	1,4	41
A 5	16,4	37,6	1,5	40
B 1	11,2	31,6	1,6	43
B 2	13,3	36,4	1,7	42
B 3	13,3	36,4	1,7	42
C 1	11,6	29,7	1,6	46
C 2	13,5	34,3	1,5	42
C 3	13,8	35,1	1,5	39

[1]) average of 4 determinations
[2]) these values were obtained before the development of the final method of analysis

Sieve fractions of some of the samples were analysed for 5–alkyl resorcinols (table 28).

TABLE 28. Influence of kernel weight on alkyl resorcinol content

	original sample	sieve fraction in mm			
		> 3,4	2,8–3,4	2,4–2,8	< 2,4
sieve fraction in % of sample weight	–	12	66	19	3
alkyl resorcinols in mg %	112	95	104	116	136
1000-kernel weight in g	32	44	34	23	14
crude protein in %	11,1	12,1	11,1	10,5	11,1

From the figures it can be concluded, that a great variance in alkyl resorcinol content occurs within one single sample of rye. It will be clear, that at least a part of this variance is caused by the variance in kernel weight. Fertilization, weather conditions and climate are known to influence 1000–kernel weight, and thus, indirectly may influence the alkyl resorcinol content. How far the factors

mentioned, also exert a direct influence on the alkyl resorcinol content of rye, is not known. More work has to be done to answer this question.

One of the consequences of the phenotypical variance in resorcinol contents in rye is, that in testing rye varieties, the alkyl resorcinol content has to be judged in relation to the kernel weight, and that it is only warranted to compare rye varieties grown under the same conditions.

CHAPTER IX

SOME EXPERIMENTS ON THE INFLUENCE OF NATURAL AND SYNTHETIC RESORCINOL DERIVATIVES ON RATS

1. Introduction

As shown in chapter VI, the growth inhibitory substance *a* is composed of a number of 5–alkyl resorcinols, accompanied by a certain percentage of 5–alkenyl resorcinols. Although the harmful effect of the mixture was demonstrated convincingly, the question arises, whether the different compounds of the mixture exert the same activity or not. Special interest has to be paid to this question, because according to Wenckert *et al* (1964) wheat also contains a mixture of different 5–alkyl resorcinols. According to the gaschromatographic analysis (table 23), the average chain length of the rye resorcinols is shorter than that of wheat resorcinols. This difference and/or a lower percentage of resorcinols in wheat may account for the fact that wheat is considered to be a better feed for pigs and poultry than rye.

Although Wenckert *et al* did not find alkenyl resorcinols in wheat, it was shown in chapter VI, that crude resorcinol preparations from rye, as well as those from wheat, probably contained 15–18% alkenyl resorcinols. Although it was not possible to prove with certainty that alkenyl resorcinols were responsible for the hydrogen uptake measured (see chapter VI) the effect of unsaturated resorcinol derivatives should not be underestimated. The work of Haack (1940) has clearly shown, that the allergenic properties of cardol and related compounds disappeared at saturation of the side chain.

To find an answer to the questions whether chain length or unsaturation play a part in the growth inhibitory effect of rye resorcinols on rats, some feeding experiments were conducted to test the effect of natural and synthetic as well as hydrogenated natural resorcinol derivatives.

2. Feeding experiments

The purified pentadecyl resorcinol, crude preparations of rye and wheat resorcinols and hydrogenated rye and wheat resorcinols, were prepared as described in chapter V and VI.

In view of the incidental anomalies observed in the rats receiving the control diet described in table 9 (see chapter III), the basic C.G. diet was used (table 29).

In experiment nr. 12 three groups of 10 male rats each, with an average bodyweight of 75 g were kept for 5 weeks on diets containing 970 g C.G diet, made up to 1000 g with:

30 g peanut oil	for diet I
30 g rye oil fraction (see exp. 8)	for diet II
25,5 g peanut oil and 4,5 g pentadecyl resorcinol	for diet III.

TABLE 29. Composition of the 'C.G.' basic diet

component	g	salt mixture	in g
maize meal	4875	$FeSO_4$.7aq	65
wheat meal	1500	$FeNH_4(SO_4)_2$.12 aq	42
casein	675	$ZnSO_4$.7 aq	25
brewers yeast	750	$CuSO_4$.5 aq	6
NaCl	37,5	$MnSO_4$.5 aq	6
$CaCO_3$	37,5	$NiSO_4$.7 aq	2
milk powder	1000	$CoSO_4$.7 aq	1
butter	100	KBr	5
salt mixture	3,5	NaF	2
0,5% tocopherol acetate		Na_2MoO_4	1
solution in peanut oil	37,5	Na_2SiO_3	100
		$Na_2B_4O_7$.10 aq	1
		KJ	0,1
		As_2O_3	0,05

Because at the start of this experiment the analysis method for 5–alkyl resorcinols had not yet been developed, the percentage of 5–alkyl resorcinols in diet II, based on the columnchromatographic separation (chapter V. 2), was valued at 0,25–0,30%. The pentadecyl resorcinol content of diet III was chosen nearly two times higher, because the unpublished results of a preliminary experiment indicated a considerably less harmful effect of the pentadecyl resorcinol. These results were confirmed by the results of experiment 12 (table 30). According to the Wilcoxon-test the control animals (diet I) grew significantly better than the other animals, whereas the difference between the animals on diet II and III was not significant.

TABLE 30. Exp. nr. 12: Average growth and consumption in g of rats on diets containing rye resorcinols and pentadecyl resorcinol

growth after	diet		
	I	II	III
1 week	23	9	10
2 weeks	51	31	27
3 weeks	81	53	46
4 weeks	105	77	71
5 weeks	130	99	94
consumption after 5 weeks	509	406	401

This means that 1,5 to 1,8 times more pentadecyl resorcinol than rye resorcinols are needed to obtain the same growth inhibitory effect.

The inhibitory effect of wheat and rye resorcinols were compared in experiment nr. 13. Both the preparations were tested at levels of 0,15 and 0,25% in the diet (970 g C.G. diet + 30 g peanut oil). Five groups of 10 male rats of appr.

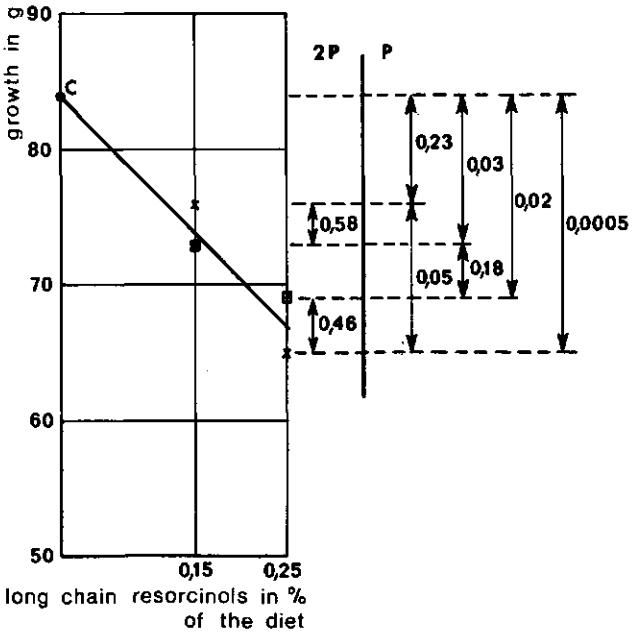


FIG. 9. (Exp. 13) Influence of different amounts of rye resorcinols (□) and wheat resorcinols (X) on the growth of rats.

42 g each were used for this experiment which lasted 25 days. The results are given in fig. 9. Growth of the rats on the highest doses of wheat and rye resorcinols was significantly lower than on the control diet ($P < 0,05$). The mutual differences between rye and wheat resorcinols (as measured by the growth of the rats) were not significant (2 P = 0,58 and 0,46 at the 0,15% and 0,25% level respectively).

Because no difference in harmfulness was observed between rye and wheat resorcinols it was considered permissable to combine rye and wheat resorcinols and to compare the growth inhibitory effect of this mixture (1:1) with the effect of the (1:1) combined hydrogenated rye and wheat resorcinols. In exp. nr. 14 four groups of 10 young male rats each, with an average weight of 42,8 g were fed for 21 days on the C.G. diet with 3% peanut oil. In the experimental diets 0,25% peanut oil was replaced by the mixed grain resorcinols (diet II), hydrogenated mixed grain resorcinols (diet III) and pentadecyl resorcinol (diet IV).

TABLE 31. Exp. nr. 14: Growth of rats on diets containing grain resorcinols (II), hydrogenated grain resorcinols (III) or pentadecyl resorcinol (IV)

growth in g after	diet			
	I	II	III	IV
1 week	22,1	15,3[1])	15,7[1])	16,7[1])
2 weeks	55,6	40,1[1])	40,1[1])	47,2[1])
3 weeks	88,2	66,6[1])	69,6[1])	80,8[2])

[1]) difference with I highly significant ($P < 0,01$)
[2]) difference with I nearly significant ($0,1 > P > 0,05$)

As can be seen from table 31, the difference between the growth of the rats on the diets II and III is only small and not significant (2 P = 0,76). This indicates that hydrogenation of grain resorcinols did not decrease their growth inhibitory effect. The growth of the animals on diet IV again confirmed the earlier finding that pentadecyl resorcinol is approximately half as active as grain resorcinols.

3. Discussion

From the results of the exps. nr. 12 and nr. 14 it can be concluded that the synthetic pentadecyl resorcinol inhibits the growth of rats, but is less harmful than the resorcinol derivative occurring in rye or wheat. Although this lower toxicity might be due to the lower chain length, or to the fact that the side chain of pentadecyl resorcinol is saturated, the results of exp. nr. 14 have shown, that saturation of the side chain of the alkenyl resorcinols occurring in rye and wheat did not alter the growth inhibitory effect of the grain resorcinol. Thus it is concluded, that the lower growth inhibitory effect of pentadecyl resorcinol is due to the shorter chain length of the pentadecyl resorcinol.

In view of this result it would have been expected, that wheat resorcinols, with a longer average chain length than rye resorcinols, would exert a more pronounced growth inhibitory effect than rye resorcinols. According to the results of exp. nr. 13 however, a difference in growth inhibition betweeen rye and wheat resorcinols is highly improbable.

In comparison to rye, the higher valuation of wheat as a feedstuff for pigs and poultry must be due to differences in resorcinol content. The column chromatographic separation of the grain resorcinols, used for the experiments 13 and 14 already showed, that rye oil yielded considerably more resorcinols than wheat oil.

Because the oil fractions were derived from wheat and rye bran, and data on the extraction rates of the mill were not available, it was difficult to estimate the actual difference in resorcinol content of wheat and rye. Accepting comparable extraction rates for wheat and rye, it could be calculated, that the rye used for these experiments contained appr. 2 times more alkyl resorcinols than wheat.

CHAPTER X

SOME EXPERIMENTS ON THE MODE OF ACTION OF ALKYL RESORCINOLS ON RATS

1. THE INFLUENCE OF THE AGE OF THE RATS

During the first feeding experiments with acetone extracted rye and barley oil, it was observed that in all probability the growth depression caused by the rye oil was not due to a lower digestibility of the rye oil, but to a decreased consumption of the rye oil diet (see chapter III. 2, exp. nr. 4).

Although a number of investigators hold the bitter taste of rye responsible for the lower food consumption, VAN WIERINGEN concluded that this probably was not the case (see chapter I). The death rate in the experimental group in a number of experiments (4, 6, 8, 9) also indicated the occurrence of a toxic substance in rye. Because, however, young weanling rats are very susceptible to suboptimal conditions, it could not be assessed with certainty that alkyl resorcinols, isolated from rye, were toxic. Because it was of interest to know whether rats could become accustomed to (the taste of) rye oil and whether older rats also were susceptible to rye oil or not, the animals of experiment nr. 4 were used for a further experiment (exp. 4a). At the 17th day of exp. nr. 4 the groups of animals receiving maize oil or acetone treated maize oil, were changed over to diets containing rye oil and barley oil respectively. As can be seen from the results

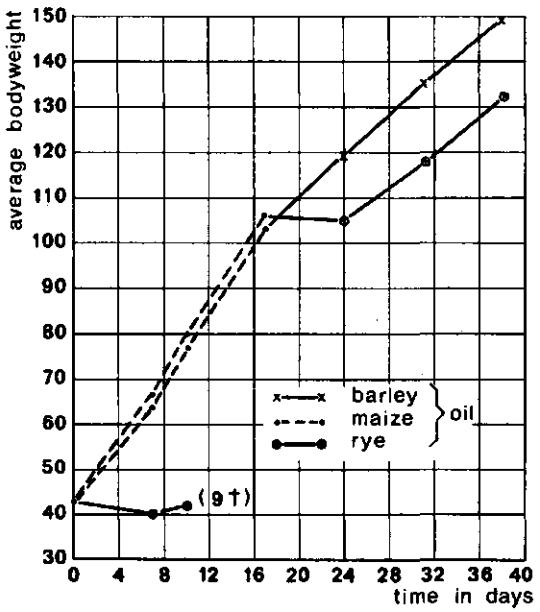


FIG. 10. (Exp. 4a) Influence of rye oil on the growth of differently aged rats

given in fig. 10, the rye oil animals did not grow at all during the first week (= 17–24 days) but afterwards showed a recovery and grew at appr. the same rate as the barley oil animals. In the period of recovery (24–38 days) food consumption and food efficiency ratio were comparable to those of the barley group. Thus it can be concluded that older rats with a bodyweight of appr. 100 g can become accustomed or adapted to a diet with rye oil as applied in exp. nr. 4, whereas the majority of weanling rats of appr. 40 g bodyweight will succomb. The recovery of the older animals indicates, that rye resorcinols in the concentration used in exp. nr. 4 are not directly toxic to rats of appr. 100 g bodyweight. The death of the weanling rats may be due to a direct toxic effect, but is caused more likely by the fact that the weanling rats, debilitated by a period of decreased food consumption, starved or died of secondary causes.

2. Study on the palatability of rye oil

To find out whether rye oil decreased the appetite of rats by its bitter taste or by any harmful effect on the metabolism of the animals exp. nr. 15 was carried out. In this experiment two groups of 20 young male rats with an average bodyweight of 48,2 g were kept fasting for the first 24 hrs after weaning. After this the two groups of separately housed animals received the basic diet described in table 9 with maize oil or maize oil and rye oil fraction 7 (exp. nr. 8). Food consumption was measured after 2 and 24 hrs.

Table 32. Exp. nr. 15: Influence of rye on food consumption

diet	oil in % of diet		total food consumption in g (20 rats)	
	maize	rye	2 hrs.	24 hrs.
I	10	–	8	91
II	7	3	17	60

As can be seen from the results in table 32 in the first 2 hrs the animals kept on diet II consumed even more than the animals of the control group. Thus it can be concluded that 'at first sight' the rats did not have any objection to the taste of the rye oil diet. After 24 hrs however, the consumption of the rats on the control diet was significantly higher than that of the animals on the rye oil diet. The fact that the intake inhibitory effect of rye oil becomes manifest in the period between 2 and 24 hrs indicates, that the food consumption was not caused by a bitter taste of the rye oil.

To obtain further information the feeding trial was continued for 14 days with 20 animals per group. The other rats were changed over to the basic diet without oil. Maize oil or the maize oil – rye oil mixture, amounting to 10% of the daily food consumption, was administered once a day by stomach tube.

As can be seen from the figures in table 33 the animals on the diets I S and II S received relatively somewhat more oil than the normally fed animals, but

TABLE 33. Exp. nr. 15: Food consumption and growth over 14 days of rats on diets with maize oil and rye oil administered in different ways

diet	number of surviving animals	oil ratio: maize – rye	actual oil intake in % of diet	average food consumption oil inclusive g	average growth in g
I N[1])	8	10:0	10,0	108	24
I S[2])	7	10:0	10,4	114	29
II N[1])	5	7:3	10,0	77	3
II S[2])	4	7:3	10,4	87	11

[1]) N = normal diet with 10% oil
[2]) S = oil free diet; oil administered by stomach tube

the differences were small enough to permit comparison of the 'N' and 'S' group. Although food consumption and growth of the 'S' animals were somewhat higher than of the corresponding 'N' animals the differences were not significant. The differences in growth and consumption between I N and II N and those between I S and II S were significant however ($P < 0,05$).

Considering that the rats on the diets I S and II S had free admittance to the same oil free basic diet it will be clear that the difference in food consumption between these groups of animals cannot be ascribed to the taste of rye oil. Thus it must be concluded, that the rye oil (resorcinols) exerts an adverse metabolic effect, resulting in a decreased food consumption.

CHAPTER XI

INFLUENCE OF RYE AND RYE OIL ON PIGS

As mentioned in chapter I both pigs and rats are susceptible to overfeeding with rye. Although the 5–n–alkyl resorcinols, isolated from rye oil proved to be the active agent, responsible for the lower growth results of rye fed rats, this did not implicate that the effect of rye on pigs is caused by the same agent. For this reason it was considered necessary to investigate the influence of 5–alkyl resorcinols on pigs.

For lack of equipment to produce enough 5–alkyl resorcinols from rye bran, it was not possible to carry out this experiment with rye resorcinols. The use of pentadecyl resorcinol was less desirable however, because according to the results of the experiments 12 and 14, the growth inhibitory effect of this compound was only 50–60% of that of rye- or wheat resorcinols. Moreover the available pentadecyl resorcinol was of a rather poor quality and needed chromatographic purification. This formed an insuperable handicap for large scale production. Therefore it was decided to compare the growth inhibitory effect of rye oil and rye with that of a control ration with barley. In the same experiment the influence of a second variety of rye and the influence of storage of rye were investigated.

1. EXPERIMENTAL[1])

Twenty farrows of 'VNL' piglets were evenly distributed over 10 groups in such a way that each group contained 5 male and 5 female animals, and that the

TABLE 34. Composition of the rations

basic ration (in % of total ration)			
component	%	component	%
maize meal	27,5	vitamin B prep.	1,0
soybean meal	10,0	vitamin A–D_3 prep.	0,05
fish meal	4,0	mineral mixture	1,5
herring meal	4,0	NaCl	0,2
lucerne meal	2,8	$MnSO_4$	0,002
		$ZnSO_4$	0,005

ration nr.	added to basic ration (in % of total ration)	
I	barley meal	50
II	rye meal (variety 'D', 1964)	50
III	rye meal ('Petkuser', 1964)	50
IV	rye meal ('Petkuser', 1965)	50
V	barley meal	49,15
	rye oil	0,85

[1]) Thanks are due to Dr. Ir. P. VAN DER WAL, director of the 'Institute of Agricultural Research of Biochemical Products' Wageningen, and his coworkers, for carrying out this experiment.

average bodyweight per group was 16,2 kg. The composition of the rations is given in table 34.

Starch equivalent and digestible crude protein values of these rations were 70 and 18 respectively. The feeding trial was carried out from medio september till end november. The rations, mixed with water (meal:water = 1:2), were given twice a day. It was tried to restrict the food consumption to approximately 80% satiation, aiming at an optimum feeding scheme (van der Wal, pers. comm.), limiting at the same time the feeding time to approximately 10 minutes per meal. Food consumption was measured twice a day per sty of 10 piglets. The animals were weighed individually at 3, 6 and 9 weeks after the start of the experiment.

2. Results and conclusions

During the experiment 58 cases of diarrhoea occurred and 6 animals died of which two in the control group (ration I). A correlation between ration and number of cases of illness could not be established, and no symptoms of overfeeding with rye, like skin irritation or 'branderigheid' were observed. Due to the relatively high number of scour-cases in the first weeks, the food consumption had to be restricted below the planned level. Therefore the growth results of all groups were lower than expected.

Table 35. Average growth and food conversion of barley and rye fed pigs after 9 weeks

ration	growth		food conversion
	kg/9 weeks	g/day	
I	31,7	503	2,35
II	28,2	448	2,54
III	27,7	440	2,54
IV	27,9	443	2,53
V	28,3	449	2,50

As can be seen from table 35, the differences in growth and food conversion between the groups II, III, IV and V were extremely small. The average growth of the animals of these groups was 11–12% lower than the growth of the control animals. However the differences between each of these groups and the control were not significant ($P = 0,10$). Since rations II–V all contained 50% rye or an equivalent amount of rye oil it was thought permissable to calculate the significance of the differences in growth between these groups together and the control group. This difference proved to be highly significant ($P < 0,01$).

From this experiment it can thus be concluded that rations with 50% rye caused a growth depression of 11–12% as compared to the same ration with 50% barley, which is in full agreement to the results obtained by others (see chapter I). It will be clear that the differences in harmfulness between the two varieties and between freshly harvested and stored rye are extremely small and can be neglected.

The most important conclusion which can be drawn from this experiment is that rye oil caused a growth depression quite comparable to that of an equivalent amount of rye.

SUMMARY

The cause of the decreased food intake and lower growthrate of animals fed on rye was investigated. By feeding experiments with rats it was proved that the causative agent was petrolether – and acetone soluble. The growth inhibitory, substance, which could be concentrated in part of the oilfraction by means of solvent fractionation and isolated chromatographically, could be identified as a mixture of 5-n-alkyl resorcinols with odd numbered side-chains of 15–23 C-atoms, and of smaller amounts of 5-alkenyl resorcinols.

The same group of compounds was found in wheat by WENCKERT *et al.* (J. Org. Chem. 1964, 29, 435). No difference in growth inhibitory effect could be detected between rye- and wheat resorcinols. The effect of synthetic 5-n-pentadecyl resorcinol amounted to only a 50–60% of that of the grain resorcinols, however. It appeared, that this could not be due to the occurrence of alkenyl resorcinols in wheat and rye, because hydrogenation of the grain resorcinols did not alter the growth inhibitory effect.

A fluorometric method for the analysis of 5-alkyl resorcinols was developed. By means of this method and of thin layer chromatography the alkyl resorcinols could be localized in the pericarp. As a consequence of this, the resorcinol content of rye is proportional to the surface area of the kernel and thus dependent on kernel size.

Feeding experiments with rats showed, that young animals are more susceptible to grain resorcinols than older ones. It could be proved, that the decreased food consumption of resorcinol fed rats was not caused by any unappetizing taste of rye oil or rye resorcinols.

The growth of pigs on rations containing 50% rye oil or an equivalent amount of rye oil, was 11–12% lower than on a 50% barley ration. No differences in harmfulness could be detected between fresh and one year old rye.

SAMENVATTING

Een onderzoek werd ingesteld naar de oorzaak van het minder goed groeien van varkens en kippen op rantsoenen waarin veel rogge voorkomt. Na een globaal overzicht van de literatuur (Hoofdstuk I) worden in Hoofdstuk II voederproeven met ratten beschreven, waarin getracht werd door destillatie en extractie de eventueel aanwezige groeiremmende stof uit de rogge af te zonderen. Aanwijzingen werden verkregen, dat de groeiremmende stof met petroleumaether extraheerbaar was. De extractie met petroleumaether leidde echter ook tot een minder goede groei van de ratten (Hoofdstuk III), zodat werd overgegaan tot extractie met aceton, hetgeen geen nadelig effect had op de proefdieren. Op deze wijze kon worden aangetoond, dat het acetonextract van rogge de groei van ratten nadelig beïnvloedt, en dat deze groeiremming praktisch recht evenredig is met het percentage rogge-olie in het rantsoen.

In Hoofdstuk IV zijn een aantal proeven beschreven, waarin getracht werd de rogge-olie te splitsen in een schadelijk en een niet schadelijk deel. Het verzepen van rogge-olie leidde tot onduidelijke resultaten, deels veroorzaakt door de invloed van het voeren van vetzuren aan de ratten in plaats van olie, deels doordat de groeiremmende stof zich waarschijnlijk verdeelde over de vetzuren en het onverzeepbare deel. Een beter resultaat werd bereikt met het neerslaan van een deel van de rogge-olie uit een oplossing van rogge-olie in methanol. De groeiremmende stof bleek bij + 2 °C in methanol oplosbaar te zijn, terwijl bij – 10 °C het oplosbare en het niet oplosbare deel een bijna gelijke remming van de groei van ratten te zien gaven.

Met behulp van dunne laag chromatografie kon in Hoofdstuk V worden aangetoond, dat een stof welke met vanilin-fosforzuur rood kleurde, en welke 'stof *a*' werd genoemd, hoogstwaarschijnlijk verantwoordelijk was voor de groeiremmende werking van de rogge-olie. Door toepassing van kolomchromatografie over silicagel kon 24 gram van deze stof *a* worden afgezonderd uit de rogge-olie. In een vergelijkende voederproef werd de werking van deze stof vergeleken met die van het residu en van de oorspronkelijke rogge-olie. Hieruit bleek dat stof *a* inderdaad de enige in rogge-olie voorkomende stof was, welke bij ratten een verlaging van de groei veroorzaakte.

Door middel van instrumentele analysemethoden kon worden vastgesteld dat stof *a* een mengsel was van 5-alkylresorcinolen met ketenlengtes van 15–23 koolstofatomen, terwijl naast deze stoffen nog een zeker percentage 5-alkenylresorcinolen aanwezig was (Hoofdstuk VI). Dergelijke stoffen blijken volgens de literatuur veelvuldig voor te komen in de Anacardiaceeën, en ook in de aan rogge verwante tarwe. Dit laatste noopte tot een uitbreiding van het onderzoek tot tarweresorcinolen. De gemiddelde lengte van de zijketen bleek bij de tarweresorcinolen iets groter te zijn dan bij de roggeresorcinolen. Tarwe- en roggeresorcinolen bleken een ongeveer gelijk gehalte aan alkenylresorcinolen te bevatten.

Verder bleek uit proeven beschreven in Hoofdstuk IX, dat het groeiremmend

effect van rogge- en tarweresorcinolen even groot was. Pentadecylresorcinol daarentegen bleek een geringer effect te hebben. Hydrogenatie van, in de mengsels voorkomende, alkenylresorcinolen leidde niet tot een vermindering van de groeiremmende werking, zodat moet worden aangenomen dat de ketenlengte van alkylresorcinolen van invloed is op het groeiremmend effect ervan.

In Hoofdstuk VII wordt de ontwikkeling van een fluorimetrische microbepaling van 5-alkylresorcinolen beschreven.

Met behulp van dierproeven kon worden vastgesteld, dat, bij een uitmalingsgraad van 65%, alle alkylresorcinolen terecht komen in de maalderijafvallen (Hoofdstuk VIII). Door middel van dunne laag chromatografie konden de resorcinolderivaten nader worden gelocaliseerd in de zemelfractie, terwijl tenslotte microscopisch kon worden vastgesteld dat de met vanilin-zoutzuur kleurende resorcinolderivaten voorkomen in de pericarp. Het vermoeden dat hierdoor de korrelgrootte van grote invloed is op het gehalte aan alkylresorcinolen kon worden bevestigd via fluorimetrische analysen. Er werden verder aanwijzingen verkregen dat niet alleen de korrelgrootte van invloed is op het gehalte aan resorcinolen, maar dat dit ook door bemesting en andere factoren direct of indirect beïnvloed wordt.

In Hoofdstuk X wordt aangetoond, dat jonge ratten veel gevoeliger zijn voor resorcinolen dan oudere dieren, en dat ratten van $\pm$ 100 g binnen enige weken kunnen wennen aan relatief hoge gehalten aan resorcinolen. De geringe groei van jonge ratten, gevoerd met rogge, bleek in eerste instantie te worden veroorzaakt door een beperking van de voedselopname, hetgeen in goede overeenstemming is met waarnemingen van anderen. Uit een voederproef, waarbij de rogge-olie per maagsonde werd toegediend, bleek dat in tegenstelling tot de algemeen verbreide mening, de smaak van rogge-olie niet de oorzaak kan zijn van de slechte voedselopname.

In Hoofdstuk XI wordt een voederproef met varkens beschreven. Op rantsoenen met 50% rogge bleven varkens 11–12% in groei achter bij dieren die in plaats van rogge 50% gerst in het rantsoen ontvingen. Rogge-olie toegevoegd aan het 50 % gerstrantsoen veroorzaakte een even grote groeiremming als het 50% roggerantsoen. Geen verschillen in groeiremmende werking konden worden vastgesteld tussen overjarige rogge, verse rogge (2 maanden na de oogst) en een rogge inteelt stam.

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