Report **Oil of Mustard & Allyl Isothiocyanate** 10/20/72

REPORT

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OIL OF MUSTARD
CAS REG. NO. MX8007407

AND

ALLYL ISOTHIOCYANATE
CAS REG. NO. 000057067

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OIL OF MUSTARD

Summary-Toxicological Information

This monograph covers the pertinent toxicological literature dealing with the term mustard which subsumes the following moities:

(1) Mustard oil as expressed from mustard seeds

(2) Mustard seed meal

(3) Mustard oil (MO) -- after enzymic hydrolysis of the glucoside sinigrin, which yields allyl isothiocyanate as the main component. This liquid also seems to be known as volatile oil of mustard (VOM), or (VMO).

(4) Synthetic allyl isothiocyanate (ATC)

(5) Mustard seed (whole or ground)

Most of the literature implies that the toxic effects of any of these moities arise from the physiological action of the ATC but except where the allyl compound itself is the test substance this assumption is clearly unwarranted.

The acute oral toxicity of MO in the rat (428) was shown to be LD_{50} = 148 mg/kg (within 24 hours). ATC toxicity in the rat (207) is LD_{50} = 339 mg/kg during a 14 day observation period.

In acute oral toxicity studies in the hen, LD_{50} values were shown to be: VMO at 42 mg/kg; fine ground mustard seed at 26 gm/kg; coarse ground mustard seed at 55 gm/kg. (330). The subcutaneous LD_{50} and ATC in white mice (235) was 80 mg/kg.

Allyl isothiocyanate fed as part of a basal diet to rats at a level of 0.02 mg/kg resulted in an increased level of serum fatty acids and serum cholesterol as well as inducing some fatty degeneration in the liver. (389). ATC has a retarding effect on the growth rate when fed to young rats (7), an effect noted also when mustard seed oil was fed to chicks. (337).

Acute to subacute stomach ulcers and some liver damage were found in all rats after getting a daily dose of ATC (20mg/kg or 50mg/kg) for 20 days. (178) Other physiological effects in rats due to feeding ATC include reduced clotting time, decreased xanthine and kidney D-amino acid oxidases, and increased levels of liver succinic dehydrogenase (7). Similarly when MO is included in the diet of rats the lipid levels rise in the serum and in the liver. (546) In chicks ingesting MO as part of their diet the plasma lipids were unchanged although the plasma non-esterified fatty acids increased. (34) In the growing chick the lipogenic effects of MO seem to be similar to that of a variety of other oils, viz: coconut, sesame, hydrogenated peanut, regardless of the degree of unsaturation. Thus, for example, in the chick aorta the phospholipid content increases while the hexosamine content decreases. (37) A possible synergism may arise between MO and cholesterol when these substances are included in the basal diet (34). Not only are the several plasma lipid moities increased (34) but the hydroxyproline content of heart aorta was also found to be increased. (37)

In these chick studies cited above (34, 37) the normal cholesterol level of the test animals were unaffected by including in the diet either MO or any of the other oils. This contrasts with the results reported for albino rats fed MO or heated MO as a dietary supplement. Thus within a 4 week period the serum cholesterol rose some 40% when the MO was included and 93% when heated MO was included. (6)

The crushed mustard seed appears to be a satisfactory forage food for cows at the rate of 1.5 to 3.0 kg/day (63) although in sheep much smaller amounts 2.5 gm/kg body weight produced short term toxic effects. (330)

When fed at condiment levels to man, mustard (as do several other spices) produced an increased salivary flow and amylase activity, while the heart volume and frequency decreased. (531) When MO was used as the only food oil in the diet of 20 human volunteers the whole blood clot-

ting time and the fibrinogen content both were found to have decreased. (167)

There is evidence that MO exerts an anti-thyroid action. This is manifest even when the MO is taken into the system as a naturally occurring ingredient of the feed, eg: cabbage. Thus rats kept on a low iodine diet and cabbage for 20-50 days developed heavier thyroids and exhibited much more severe thyroid changes than rats kept on the same diet in which carrots were substituted for cabbage. The ATC component of cabbage MO may act synergistically with the other MO species so as to enhance its anti-thyroid action, since a dose of synthetic ATC 10-20 times that found in the MO of cabbage is required to produce the same thyroid changes noted above. (262)

Synergism among 3 different goitrogens including ATC was also shown (263) by introducing various amounts (singly or in combination) into male rats by stomach tube daily for 21 days. On the 21st day carrier-free ^{131}I was injected intraperitoneally. The results indicated that the greater the number of substances fed together (and the larger their doses) the greater was the antithyroidal effect. The various levels employed of the three substances were ATC 0.5 and 5.0 mg., thiocyanate 1, 2, 4, 8, 16 mg., and goitrin 50γ, 500γ.

Mustard oil and/or ATC is well known as a skin irritant; even 1% MO emulsion is an active mucosal irritant giving rise to large amounts of a serous transudate secretion. (190) Numerous studies have been made to establish the mechanism and the consequences of the irritation. Examples include:

- Electron-microscopy examination of the influence of irritants on the epidermis of mice. The changes in the epidermis of albino mice produced by mustard oil, turpentine, and cantharidin consist in the deposition of DNA on the inner nuclear membrane. (364)

- Mustard oil causes redness, miosis, and chemosis of the rabbit eye. Histamine produces the same effect but without miosis. (579)
- Direct contact of a number of different spices with the gastric mucosa for a 3-hour period resulted in some edema in the case of cinnamon, pepper, paprika, and mustard. (609)
- Successive application of MO to the skin of rabbits produces a local immunity to the irritant. Animals that on first contact with the oil showed extensive local necrosis (and required several weeks for recovery) proved to be refractory to reapplication of the irritant. (396).
- When mustard oil is applied to the skin of a dog ear, the irritation causes a decreased blood supply for 1 minute followed thereafter by an increase in the blood supply lasting an hour. MO usually causes a 50-65% decrease in oxygen consumption in the ear and a 2-5 fold increase in CO_2 output of the arterial and venous blood as determined by gasometric analysis. The condition reverses 3-4 hours later, the oxygen consumption increasing and CO_2 output decreasing. In addition, there results very marked edema and cellular accumulation. (388)
- Applying MO to the skin can have more serious consequences than simply local necrosis. This is particularly true for individuals allergic to mustard. Two cases are reported of anaphylactic shock in humans resulting from skin testing of MO which was fatal to one of the patients. Initially the two patients showed no sensitivity to MO when applied as a scratch test. However when it was injected intradermally the shock reactions appeared within 10 minutes. (437)

Closely related to skin contact investigations are inhalation studies. Such a study was carried out on 250 guinea pigs exposed to a number of gases including allyl mustard oil. Different groups of animals were exposed from 1-20 times in large glass vessels for 3-20 minutes according to the gas. 34 guinea pigs died within 12 hours and the 216 that survived were examined histologically. The principal changes produced by inhalation of any of the vapors were ulceration, necrosis, and degeneration of the epithelial cells, all kinds of degenerative changes, vascular dilation and hyperemia, epithelioid vacuoles, cyst formation, and not infrequently, hemorrhage. (440)

Allyl isothiocyanate administered into the dog(presumably by i. v. injection) has no direct constricting action of the muscles by the blood vessels but acts on the nervous centers. (373) This excitation of the central nervous system produced hyperpnea which could not be avoided even by carotid denervation or section of the vagal nerves or of the spinal cord. (374)

Intravenous injection of a 0.25 - 0.45 MO emulsion into female rabbits resulted in distinct shallowness of respiration and decrease in respiratory exchange. The emulsion was made by dispersing 1-2 drops of oil in 1 cc. of dilute gum arabic solution (228). When introduced into rats by intraplantar injection MO produced a decrease in the animals eosinophile count and depletion of the adrenals; this occurred even in the case of hypophysectomized animals. In addition, the growth of foreign body granuloma was encouraged. These reactions were also shown by injection either of croton oil, turpentine, egg white, or dextran. (172)

Mustard oil has been tested for its carcinogenic activity on XVII x C57 (black) hybrid mice by 4 different routes: (i)Cutaneous application, (ii) subcutaneous injection, (iii) intraperitoneal injection, and (iv) oral ad-

ministration. There was no evidence of malignancy due to oil at the site of treatment or in the remote organs even though the animals were observed for their complete life span of over two years.(365)

The metabolic fate of mustard in animals seems not to have been investigated and only one study was discovered concerning mustard metabolism in man. When fed to human subjects p-hydroxybenzylamine was found in the urine.(589)

OIL OF MUSTARD

Chemical Information

I. Nomenclature

A. Common names (Merck Index 8th Ed. 1968)

- Oil of Mustard, Expressed
- Volatile Oil of Mustard (after enzymic conversion)
- Mustard Oil (after enzymic conversion)

The major component, allyl isothiocyanate, is also known by the following common names (350):

- Allyl Mustard Oil
- Mustard Oil, Artificial
- Allyl Senevol
- Red Skin (Merck Index)

B. Chemical names (for the allyl component) (350)

- allyl isothiocyanate
- allyl isorhodanide
- allyl isosulfocyanate
- allyl thiocarbonimide
- isothiocyanic acid, allyl ester (Merck Index)

C. Trade names

- Mustard Seed Oil (Chemical Buyers Directory)

D. Chemical Abstracts Services Unique Registry Number

Oil of Mustard : MX8007407

Allyl isothiocyanate : 000057067

II. Empirical Formula

Mustard oil is a mixture of substances, with the major components being (164) :

Allyl isothiocyanate :	C_4H_5NS
Allyl cyanide :	C_4H_5N
Carbon disulfide :	CS_2

Allyl Isothiocyanate represents more than 90% of the mustard oil mixture.

III. Structural Formula (Handbook of Chemistry)

Allyl isothiocyanate	:	$CH_2=CH-CH_2-N=C=S$
Allyl cyanide	:	$CH_2=CH-CH_2-C\equiv N$
Carbon disulfide	:	$S=C=S$

IV. Molecular Weight

Allyl isothiocyanate	:	99.15
Allyl cyanide	:	67.09
Carbon disulfide	:	76.13

V. Specifications (164)

A. Chemical

Allyl isothiocyanate - 93% - 94%

Allyl cyanide and carbon disulfide - approx. 6%

B. Food Grade (132)

Assay: not less than 93% allyl isothiocyanate

Limits of Impurities:

Arsenic (as As). Not more than 3 ppm (0.0003%).

Heavy metals (as Pb). Not more than 40 ppm (0.004%).

Lead. Not more than 10 ppm (0.001%).

Phenols. Passes test.

C. Official Compendia

Food Chemicals Codex. 1st Edition. 1966.

VI. Description

A. General characteristics

Oil of mustard is a colorless or pale yellow, strongly refractive liquid, having a very pungent, irritating odor and an acrid taste. It is miscible with alcohol, with ether, and with carbon disulfide. It is optically inactive. (132)

B. Physical Properties (164)

Specific Gravity at 15°	1.014 to 1.022, occasionally as high as 1.030
Optical Rotation	± 0°
Refractive Index at 20°	1.52681 to 1.52908
Boiling Range at 760 mm	Mostly between 148° and 154°

Solubility	Soluble in 160 to 300 parts of water. Soluble in 7 to 10 vol. of 70% alcohol; in 2.5 to 3 vol. of 80% alcohol; in 0.5 vol. of 90% alcohol. Clearly miscible with ether, amyl alcohol, benzene and petroleum ether in all proportions.

Oil of Mustard, Expressed, consists chiefly of the glycerides of oleic acid and other fatty acids, including arachidic. It is straw colored or brownish yellow or greenish brown liquid, insoluble in water, slightly soluble in alcohol and miscible with chloroform, ether, and petroleum ether. (164)

The precursor of allyl isothiocyanate is a glucoside, sinigrin (potassium myronate), which, in the presence of water and a ferment (viz., myrosinase), is hydrolyzed, yielding allyl isothiocyanate, d-glucose, and potassium bisulfate. (164)

C. Stability in containers, animal feeds, etc.

Allyl isothiocyanate is very unstable. It is decomposed under influence of air, light, water, metals, etc., and even alcoholic solutions are unstable. (350)

VII. Analytical Methods

Identification and Quantitation

- GLC of allylisothiocyanate in mustard seed (14)
- GLC - identification by fatty acid composition (335)
- Separation of volatile and non-volatile MO fractions and their UV identification (265)
- Colorimetric test for hydrocyanic acid in MO. (310)
- Electrophoretic (acrylamide gel) identification. Distinguishing among mustard seed varieties by characterization of their protein components. (628)

VIII. Occurrence and Levels

A. Plants:

In spices, the term mustard refers to the seeds of two different plants of the same family — namely, Sinapis alba L., from which is obtained white mustard seeds, and Brassica nigra (L.) Koch (also named Sinapis nigra L.) from which is obtained black mustard seeds. The former is commonly known as yellow mustard and the latter as brown -- understandable terminology when the color of the respective seeds is considered.

Both plants belong to the Cruciferae, the name being derived from the crosslike figure formed by the four petals of the flower, or Mustard family. (342a)

Natural allyl isothiocyanate, also known as volatile mustard oil is obtained by maceration with water and subsequent distillation of the dried ripe seed (free from fixed oil) of Brassica nigra (Linne) Koch, or of Brassica

MUSTARD OIL

Biological Data

Rat	oral	ATC *		339(c)	207
Hen	oral	VOM *	25	42	330
Hen	oral	mustard seed coarse ground		55gm.	330
Hen	oral	mustard seed fine ground		26gm.	330
Hen	oral	whole mustard seed	105gm(d)		330
White mice	s.c.	ATC		80	235

(a) Tolerated dose=highest daily oral dose given over an 8 week period without causing noticeable injury to animals.

(b) Lethality within 24 hours.

(c) Lethality within 2 weeks.

(d) Administered in a single dose.

* ATC = allyl isothiocyanate
VOM = volatile oil of mustard

II. Short-Term Studies

Rats fed a diet containing ATC (0.02 mg/kg body weight)showed an increased level in serum fatty acid esters and in cholesterol. Histologically some fatty degeneration of the liver was noted but this was most marked when the ATC was fed in combination with ethyl alcohol (1.5 milliliters of a 20% solution per hundred grams animal weight.(389). ATC is reported to have a deleterious effect on growth when fed to young rats. In these animals it was observed that the clotting time was reduced, the liver succinic dehydrogenase was increased, and both xanthine oxidase and kidney D-amino acid were significantly decreased.(7)

Two groups of rats (5M and 5F each) received a daily dose (by stomach tube) of 20 mg/kg and 50 mg/kg ATC respectively for 20 days. At both levels of feeding, organ and tissue examination showed the following pathology: (178)

Stomach: macroscopic -- non-glandular part of the stomach was thickened, with lining occasionally roughened; microscopic --slight to moderate epithelial hyperplasia of the non-glandular part of the stomach, with acute to subacute ulcers from 2 to 6 1/2 mm across observed in all animals.

Liver: microscopic -- minor inflammatory foci.

Rats were fed a basal diet supplemented with either of a number of oils including MO. MO had a lipid lowering effect on serum but this was apparent only during the early part of feeding; subsequently the level showed a steady upward trend. MO also produced consistent and progressive increases in the liver lipid level.(546)

The overall nutritive contribution of (expressed) MO seems to be limited to its role as a fat source. Thus feeding tests with rats show that mustard oil: (a) is entirely destitute of vitamin A; (b) tends to inactivate vitamin A in other food articles given along with it; (c) but, as fat, is as nutritive as other fats, provided sufficient vitamin A is supplied in other articles of food. (60)

In contrast to the MO, mustard seed meal (treated to remove the sharp flavor) appeared to be a satisfactory protein source and produced no apparent toxic factors when fed to rats extending over two generations (533)

In those areas where the mustard plant is indigenous numerous studies have been carried out to establish the toxicity and possible efficacy of the meal as a feed source for farm animals especially chicks. Thus in one study meal containing 10% crushed S. arvensis seed was fed daily to 20 chicks 3 weeks old. Toxic signs appeared after 90 days and by the 122nd day 9 chicks had died. (330)

Eight groups of 40 unsexed day-old New Hampshire chicks were fed for a period of 8 weeks on a basal diet containing 19.5% animal protein. The control group received no vegetable protein, 3 groups were fed increasing amounts of sunflower seed oil meal, and 4 groups were fed increasing amounts of mustard seed oil meal. Results showed that 1/3 of the animal protein could be replaced with sunflower seed oil meal without affecting growth, but 2.8% or more of mustard seed oil meal resulted in significantly lower body weight by the 8th week. (337)

The possibility of synergism between MO and cholesterol was studied by including in the feed of growing chicks MO (10% of diet) with

or without 1% cholesterol for 8 weeks. MO alone increased only plasma non-esterified fatty acids but did not effect any change in plasma lipids. But MO and cholesterol yielded increases in total plasma cholesterol, β-lipoprotein, cholesterol, % β-lipoprotein triglycerides, and non-esterified fatty acids. The extent of hypocholesterolemia did not seem dependent on degree of saturation of the MO. (34)

Groups of growing chicks were fed one of several oils including MO as 10% of their diet with or without 1% cholesterol for 8 weeks. Portions of the aorta were analyzed for cholesterol, phospholipids, hexosamine, and hydroxyproline. In addition the distribution of elastic tissue, acid micopolysaccharides, & lipids were also determined using histochemical methods. It was found that:(37)

- Cholesterol with or without oils increased both cholesterol and phospholipids in different parts of the aorta.
- Oils without cholesterol caused an increase in phospholipids but no change in cholesterol.
- Hexosamine content in the aorta decreased regardless of whether cholesterol was fed, which implies a concomitant decrease in acid mucopolysaccharides.
- Hydroxyproline content of aorta increased when oils and cholesterol were fed, indicating that the arterial collagen had increased. This last seemed to be proportional to the degree of hypercholesterolemia.
- Severity of changes were not related either to the cholesterol level in plasma or to the degree of unsaturation of oils.

The crushed seed appears to serve as satisfactory feed for larger farm animals. For example, crushed mustard seed was fed as forage to cows at the rate of 1.5 kg/day dry up to 3 kg/day. Satisfactory nutrition of animals was maintained with no effect on milk fat content. (63) In another study pregnant sheep were administered a daily dose of up to 80 gms. of ground mustard seed as part of their feed concentrates. No toxic

effects were noted. However, a single dose of 2.5 gr. crushed seed per kg. bodyweight did produce "transitory ruminal tympany in some sheep". When the whole green plant was fed in large amounts (30 to 35 kg.)some sheep had "brief ruminal atony and tympany". (330)

Observations of the short term physiological effects due to mustard (among other spices) fed as a condiment in the test diet were made (presumably) on human subjects. The spices studied were (a) citron, (b) curry, (c) red pepper, (d) mustard, and (e) sugar. It was noted that the increase in salivary flow was about the same magnitude for all of the spices, while the increase in amylase activity followed the order b > a > e > d > c. The neuraminic acid levels remained unchanged throughout the test period.

III. Long Term Studies

MO, sesame, or coconut oil with and without added cholesterol were included in the diet fed to rhesus monkeys for 9 months. The animals were then sacrificed and total lipids, and levels of cholesterol, and phospholipids were determined in various organs. Compared with normal values, in those animals fed diets containing MO, diminished amounts of cholesterol deposits were found in the thoracic and absominal aortas, in the brain, and in the skin, while increased levels of those substances were found in the liver. In contrast, sesame oil-fed animals showed increased cholesterol deposition in the thoracic and abdominal aortas, and coconut oil feeding caused an increase in liver lipids. Comparative effects among the oils was not as clear when cholesterol was added to the diet; the degree of saturation seemed not to be a factor. (35) In a similar study Rhesus monkeys were fed cholesterol and either MO, sesame, or coconut oil in their diets for 6 months. Periodic observation made in plasma lipids showed that all oils raised plasma cholesterol level (probably by facilitating intestinal absorption). Again no relation noted to the degree of unsaturation of the fats. (36)

ATC fed to 3 groups of rats (5M & 5F) for 26 weeks produced no toxic effects. The dosage level for each of the groups was 10000, 2500, and 1000 ppm respectively. (178)

Twenty human volunteers were fed diets made up with either of six different oils including MO so as to establish the effect of these oils on the blood parameters. In the case of MO the following results are reported: (167)

- Whole blood clotting time decreased

- Stypven time (plasma) - no change
- Platelet adhesiveness-increased (after 2 hours).
- Blood fibrinolysing property (fibrinolysin titer) no effect.
- Fibrinogen content-decreased
- Platelet content - no change
- Serum cholesterol - no effect

Studies on the long term effect of mustard fed as a condiment were made on rats. These animals were kept on diets containing 2% pepper, 1% mustard and 1% ginger, or 2% pepper 1% mustard and 1% ginger, and all developed hypertension within 2-3 months. The mustard and ginger diet maintained hypertension for a year while the diet containing all three of the spices resulted in irreversible hypertension. Histopathologic findings on the kidneys after 1 year were essentially negative. (382)

Biochemical Aspects

I. Breakdown

(none)

II. Absorption - Distribution

(none)

III. Metabolism and Excretion

Normal human subjects were maintained on "plant-free" diets for 72 hours and then fed large amounts sequentially of several different fruits, vegetables, and flavoring agents. Mustard was found to produce the exogenous urinary amine p-hydroxybenzylamine. In contrast, orange resulted in the appearance of synephrine while vanilla extract produced 3-methoxy-4-hydroxybenzylamine. (589)

IV. Effects of Enzymes and Other Biochemical Parameters

Rabbits weighing 1.5 kg each were gavaged with 8 ml. of MO (among other oils). The extent of lipemia and its clearing by lipase was detected colorimetrically. Enzyme activity was high in clearing MO lipemia compared to butter, ghee, Dalda, and cod liver oil. (75)

It was observed in in vitro experiments that up to 10^{-2} M allyl isothiocyanate depresses guinea pig liver slices as much as 50% in a linear relation independent of glucose in substrate. ATC at the 10^{-4} — 10^{-3} M level increases respiration up to 21% by stimulating CHO oxidation by both cell and substrate without stimulation of fat oxidation. At the 10^{-2} M level ATC inhibits fat and substrate CHO oxidation. (44)

V. Drug Interaction

(none)

VI. Consumer Exposure Information

(none)

OIL OF MUSTARD

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XIII. Pharmacological and Toxicological Effects of Food Components — Effets pharmacologiques et toxicologiques des composants alimentaires — Pharmakologische und toxikologische Effekte von Nahrungsbestandteilen

Biochemical Effect of Allylisothiocyanate

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Mustard oil has been extensively used in East Pakistan, West Bengal and Behar as the principal cooking fat from time immemorial. The seeds obtained from the plant *Brassica nigra, Brassica napus* and *Brassica juncea,* after extraction, contain the esters of isothiocyanic acid, and commonly known as mustard oil. Mustard oil occurs in the form of a glucoside known as sinigrin which in the presence of the enzyme myrosin and water, is hydrolysed into mustard oil, D-glucose and potassium acid sulphate.

The seeds of mustard contain 0.66 to 1.23 percent essential oil of mustard. This oil contains 96.0 to 97.7 percent of allylisothiocyanate (AITC) [1]. AITC is a colourless oil which turns yellow. It is optically inactive and possesses a very penetrating odour, causing flow of tears. Applied to skin it produces burning sensation and causes blistering. It has also been reported that prolonged contact of AITC may cause vesication and slowly healing ulcers. The vapours are particularly harmful to lungs. Ingestion of large doses causes severe gastrointestinal inflammation and may result in circulatory collapse and death [2].

Leblova-Svobodowa and Kostir [3] have shown that the germination and growth of pea, wheat and rape was distinctly suppressed by AITC in concentration of 5.10^{-3}M. They also concluded that AITC belongs to the most powerful inhibitors of protein synthesis in these germinating seeds.

Hercus and Purves [4], in tests with *Brassica* seeds on rats, found goitrogenic activity in rape seeds, cabbage seed, steamed white mustard seed and steamed black mustard seed. Goitrogenic activity of *Brassica* seeds was also confirmed by Kennedy and Purves [5].

In another experiment, Langer and Victor [6] showed that allylisothiocyanate, administered in single doses of 2 to 4 mg. per 100 gm. body weight, depressed significantly the radioiodine uptake by rat thyroids. The administration of AITC to rats for 60 days, in doses corresponding approximately to the total content of mustard oils in 35 to 40 gm of cabbage per day, caused changes in the thyroid similar to those following cabbage feeding. About 9 mg. of AITC is present in 100 gms. of fresh cabbage.

Considering all the effects of AITC described above the present study was undertaken to see the effect of synthetic AITC feeding on the growth, plasma constituents, blood and liver and kidney enzymes in rats.

Methods and Materials

A. Growth

A nine week experiment was conducted using rat as an experimental animal. Two groups of litter-mate 21 days old weanling rats, consisting of 4 rats in each group, weight varying from 26 to 27 gms., were selected for the study. The rats were placed individually in mesh-bottomed cages. The basal diet had the composition of whole wheat flour 34.0 %, rice flour 22.7 %, Bengal gram 15.7 %, skimmed milk 22.7 %, sodium chloride 1.5 %, calcium carbonate 1.5 % and calcium phosphate 1.5 %. Cod liver oil was added to the diet 5 drops per week. This diet contained 18 percent protein. The control group received the basal diet alone whereas the experimental group received basal diet mixed with 0.1 % AITC for 5 weeks.

During the next 4 weeks i.e. from the 6th to the 9th week, stomach feeding of the calculated amount of AITC (0.1 % of the daily intake of food) was done daily. During the 9 weeks of experimental period all the rats received diet and water *ad libitum*. Bi-weekly gain in weight of the rats was recorded for the whole period.

B. Blood clotting and prothrombin time

Two groups of adult rats, 4 in each group weighing from 200 to 246 gms. were selected for this study. All these rats received the basal diet described before.

On the day of the experiment, blood from individual rats was collected from tail. The clotting and prothrombin time was determined according to the methods described by Sabraze [7] and Quick and Smith [8], respectively. After feeding the calculated amount of AITC (0.2 mg.% of the body weight), blood was collected from tail after 4 hours, again clotting and prothrombin time was determined according to the above mentioned methods.

C. Plasma constituents and liver and kidney enzymes

For these studies, 8 adult rats weight 106 to 107 gms. were selected. They were divided into experimental and control groups randomly and kept on the same basal diet prepared previously. On the day of experiment, the experimental rats were fed AITC (0.2 mg./100 gm. body weight) and after 4 hours both the groups were sacrificed under ether. Blood was drawn from heart through oxalated syringe and was then transferred to the oxalated vacutainer tubes (Becton, Dickinson and Company, Columbus, Nebraska) and then centrifuged for 15 minutes at 3000 r.p.m. The plasma was separated and kept in deep freeze.

The liver was removed and chilled in a beaker cooled with ice for xanthine oxidase and succinic dehydrogenase assay. The kidneys were also removed and chilled too in the same way for D-amino acid oxidase assay. They were then weighed and homogenised to give a 10 % solution of liver in deionized water. The enzymatic assay of D-amino acid oxidase, xanthine oxidase and succinic dehydrogenase was started immediately by manometric technique using Warburg respirometer. The assay methods of the enzymes, succinic dehydrogenase, xanthine oxidase and D-amino acid oxidase were as described by Umbreit and Burris [9], [10], and Axelrod and coworkers [11].

Analysis of the plasma constituents, total lipid, total protein and cholesterol was started on the following day. Total lipid was estimated by the method reported by Bragdon [12] whereas cholesterol was analysed following Carr and Dexter [13]. Total protein was estimated by the method of Wolfson et. al. [14].

Results and Discussions

In Figure 1 is shown the effect of AITC on the growth of young rats. It can be seen that during the five weeks when AITC was administered mixed with diet (0.1 %), there was no significant effect. But when AITC was begun to be administered through stomach tube (0.1 % of the total intake of food), the effect became obvious. While administration through a stomach tube introduced the whole amount of AITC all at once, the administration of the substance along with the diet meant ingestion of the same in smaller amount over a longer period.

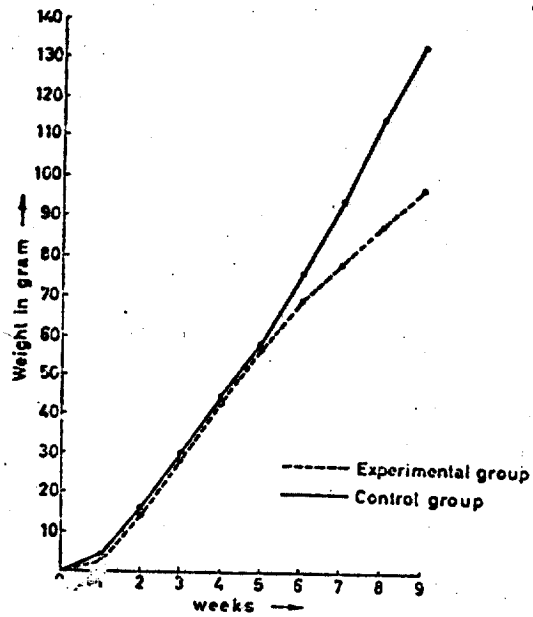

Fig. 1
The graph shows the gain in weight of rats for 9 weeks.

Table I shows the effect of AITC on blood clotting and prothrombin time. It can be seen that clotting time is significantly decreased, by almost 37 %, whereas prothrombin time has not decreased at all. Further work is in progress to find the cause of the decrease of this clotting time.

Table I. Data show the effect of allyl isothiocyanate feeding on the clotting and prothrombin time of the blood of rat.

Serial No. of rats		Clotting time (in seconds) [1] Before feeding	After feeding	Prothrombin time (in seconds) [2] Before feeding	After feeding
Group I	A	175	110	35	30
	B	175	105	36	35
	C	165	115	32	34
	D	160	110	33	30
Group II	A	165	105	32	32
	B	160	100	35	35
	C	170	110	30	30
	D	175	115	32	32

[1]) According to statistical analysis, calculated t 14.1 and 11.3 for group I and II respectively whereas theoretical t = ± 2.447. The difference is highly significant.
[2]) Similarly, calculated t = 0.25 and 0.45 respectively for group I and II whereas theoretical t = ± 2.447. The difference is insignificant.

In table II, certain plasma constituents like total lipids, cholesterol and total protein are shown. There is a tendency of increase in lipid constituents, but statistically this increase is not of any significance under the conditions of the experiment.

Table II. Data show the effect of allyl isothiocyanate feeding on the total protein, total lipid and cholesterol level of the rat plasma.

Serial No. of rats		Initial wt. of rats (gm)	Total lipid mg/100 ml plasma	Cholesterol mg/100 ml plasma	Total protein gm/100 ml plasma
Control Group	A	106.0	291.0	62.0	—
	B	140.0	198.0	53.0	4.79
	C	143.0	304.0	48.0	5.33
	D	156.0	270.0	37.0	5.81
	E	164.0	340.0	44.0	6.15
Average		141.8	330.6	48.8	5.52
Experimental Group	A	137.0	370.0	62.0	—
	B	142.0	278.0	91.0	4.44
	C	152.0	340.0	36.0	5.05
	D	156.0	450.0	68.0	6.19
	E	170.0	420.0	66.0	5.32
Average		151.4	371.6	64.6	5.25

According to the statistical analysis no significant difference was found between the control and experimental group in case of total lipid, cholesterol and total protein. (Calculated $t = \pm 1.519$, ± 1.671 and ± 0.299 respectively whereas theoretical $t = \pm 2.447$ at $p = 0.05$).

In table III, are presented results on the investigation of the effect AITC on liver and kidney enzymes.

Table III. Effect of allyl isothiocyanate feeding on the activity of D-amino acid oxidase, xanthine oxidase and succinic dehydrogenase in rat liver and kidney.

Serial No. of rats		D-Amino acid oxidase [1]) O_2 consumption in mcl/hour/gm. tissue (kidney)	Xanthin oxidase [1]) O_2-consumption in mcl/hour/gm. tissue (liver)	Succinic dehydrogenase [2]) O_2-consumption/hour/gm. tissue in mcl (liver)
Control Group	A	1295.68	677.35	495.40
	B	1904.44	695.57	896.13
	C	2246.71	1078.82	814.38
	D	2231.32	1074.35	637.58
Average		1919.53	881.52	710.87
Experimental	A	1213.94	210.97	669.92
	B	1728.61	577.42	1073.15
	C	1953.14	621.77	1587.01
	D	1952.52	641.15	916.74
Average		1711.8	512.82	1061.70

[1]) According to statistical analysis of individual pairs, there was a significant inhibition of O_2 consumption in experimental group. Calculated $t = 3.630$ and 4.483 for D-amino acid oxidase, and xanthine oxidase respectively, whereas theoretical $t = \pm 2.447$ at $p = 0.05$.

[2]) In case of succinic dehydrogenase, there was a signifikant increase of O_2 consumption calculated $t = \pm 2.458$ whereas theoritical $t = \pm 2.447$ at $p = 0.05$.

It can be seen that D-amino acid oxidase in the kidney and xanthin oxidase in the liver have significantly decreased, while there is a significant increase of the succinic dehydrogenase enzyme activity.

Summary

Effect of allylisothiocynate present in mustard seed and oil and a few other members of the Brassica family of plants on the growth of young rats has been studied and found to have deleterious effects. The same substance when fed to rats also reduced clotting time of their blood. The treated animals were also found to have increased succinic dehydrogenase in the liver, while liver xanthine oxidase and kidney D-amino acid oxidase decreased significantly.

Acknowledgment

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Extracted mustard seed groats as a concentrated dairy cattle feed.

The results of several feeding tests on milch cows.

By H. Buenger-Kiel.

In the following article H.Buenger-Kiel summarizes the reports and test results by J. Schmidt and J. Kliesch, Institute for Animal Husbanding and Genetics of Domestic Animals, Berlin; K. Richter and H. Baensch, Institute for the Feeding of Domestic Animals, Kraftborn; W. Büenger and H. Schmidt, Institute of Dairy Production, Kiel, and K. Nehring, Agricultural Research Station Seestadt Trostock.

Since more mustard seeds were grown in last spring to replace among others the great losses of winter oil seeds in the winter of 1941/1942 and consequently a greater amount of mustard seed residues had to be expected, the Office of Nutrition suggested to test again the feed properties of these residues. This seemed particularly indicated because the tests had been made in the past with expressed residues of the seeds, while at present only extracted residues are obtained. Besides, the tests and observations in the past had led partly to different results.

The mustard seeds just like rapeseed and colza contain a mild, odorless fatty mustard oil, which can be used like rape oil. Besides, all mustard seeds, both of black mustard, sinapis nigra, and of white mustard, sinapis alba, and of wild mustard, sinapis arvensis, develop when wetted with water in larger or smaller amounts a volatile, mustard oil with a pungent odor, which can cause considerable health injuries in large quantities.

According to Pott (1), the expressed residues of mustard seeds can be used as feed after treatment with steam, where the volatile mustard oil distills off, if we proceed carefully, since they can also develop mustard oil as a rule. The mere odor of mustard oil already leads in many cases to refusal of feed intake.

Honcamp (2) reports that in tests with wethers, the first samples of brown mustard seed residue were well accepted. A second sample was at first like-

wise eaten by the animals, but after the few days they lost their appetite completely. In a younger animal paralysis and diarrhea were observed.

Dr Vries (3) fed the animals white mustard seed residues as a substitute for rape oil cake. The feed was not always well digested. The test and odor of the mil was also sometimes adversely affected.

The extracted mustard seed groats to be tested were supply by Hauptvereinigung der Deutschen Getreide- und Futtermittelwirtschaft from the oil mill of Wilh. Rodrian, Bielitz, U.S. According to the consumer department of the Office of Nutrition, a part of the supply was steamed for 2 hours, another part only briefly.

For carrying out a feeding test on milch cows, the following institutes were available: Institute of Animal Husbanding and Genetics of Domestic Animals at the University of Berlin, director Prof. Dr. J. Schmidt, Institute for the Feeding of Domestic Animals, at the Prussian Research Institute for Animal Husbanding in Kraftborn, director Prof. Dr. K. Richter, and Institute for Dairy Production at the Prussian Dairy Research Institue in Kiel, director Prof. Dr. H. Buenger. In addition, the Agricultural Research Station Rostock, director Prof.Dr. K. Nehring investigated mustard seed groats and made simple feeding tests on wethers. The necessary funds were made available by the Ministry of Nutrition and Agriculture through the research agency.

For the feeding tests on milch cows the Institute of Animal Husbanding in Berlin received a product which had been steamed for 2 hours, while the Institute in Kraftborn and Kiel were supplied with the briefly steamed extracted groats. The Research Station in Rostock received groats from both, in addition mustard seeds.

The investigation of the extracted mustard seed groats showed the following values for the raw nutrients:

Table 1.

	Extracted mustard groats							Mustard grain Rostock
	Steamed for 2 hours			Steamed briefly				
	B.%	R.%	mean%	Kr.%	K.%	R.%	mean%	
Dry substance	91.79	91.68	91.70	93.5	92.12	91.26	92.29	91.66
organic substance	84.10	84.63	84.36	86.2	84.59	84.41	85.07	85.46
Raw protein	41.60	46.00	43.80	42.8	41.37	43.61	42.59	29.17
Pure albumin	36.41	36.31	36.36	38.8	--	35.88	37.34	22.33
Raw fat	0.90	2.26	1.58	0.2	0.77	1.50	0.82	22.08
Raw fiber	11.24	9.35	10.30	14.0	9.03	9.30	10.78	8.25
N-free extracted s.	30.36	27.02	28.69	29.2	33.42	30.01	30.88	25.96
Ashes	7.69	7.05	7.37	7.3	7.3	6.85	7.23	6.20

B. - Berlin; R. - Rostock; Kr. - Kraftborn; K - Kiel

With an average of about 43%, the content of raw protein is rather high, much higher than was to be expected according to a part of previous data.

For the calculation of the digestible nutrients there are no recent tests available. Schmidt used therefore the digestion values indicated in the Kellner Tables for rape oil cake. Richter determined the digestion values in special tests. Buenger used as a basis for the digestible raw protein the value of 88 indicated by Nehring, for the other nutrients the values in older tests by Honcamp.

	Extracted mustard seed groats			
	steamed for 2 hours	briefly steamed		
	B. kg	Kr. kg	K. kg	mean kg
Digestible raw protein	33.7	34.6	36.4	35.5
digestible pure albumin	29.5	--	--	--
starch value in 100 kg	58.1	58.9	61.5	60.2

On the average we obtain for the extracted mustard seed groats with a starch value of about 60 kg a content of about 35% of digestible raw protein and 30% digestible pure albumin. Extracted mustard seed groats thus prove to be one of the residues of oil production with the highest albumin content and has much more albumin than the rape oil residues, of which the rapeoil cakes have about 27%, extracted rape oil groats about 28 to 30% digestible raw protein.

The test for non-volatile mustard oils (allyl mustard oil) made in Rostock

yielded in all samples a content of only 0.04%, which seems harmless. After wetting with lukewarm water, no marked mustard oil odor was observed according to our own observations.

The feeding tests on milch cows were carried out as follows:

Institute	Test groups	Test cows	Testing time	Extracted mustard seed groats per animal and day
Berlin	2	12	79	to 2.0 kg
Kraftborn	1	6	51	to 2.2 kg
Kiel	2	12	60	to 3.0 kg

The tests showed the following results.

All institutes report unanimously that the test cows had no trouble getting used to the extracted mustard seed groats. Even portions of 2 kg and even 3 kg per cow a day were well digested by the cows after a short break-in period, namely when the mustard groats were mixed with other feeds, like dry chips. Unmixed, the cows accepted the mustard seed groats more hesitatingly. The extracted mustard seed groats proved a very well tolerated feed. No harmful effect on the health of the cows were observed.

In Rostock sample feedings on wethers were made with both types of extracted mustard seed groats, administering 200 g for one week, in addition to the usual feed. The extracted mustard seed groats were taken without any difficulties. Special symptoms were not observed.

The effect of the extracted mustard seed groats on the milk output was identical in all three tests, corresponding to the conent of digestible nutrients. The milk output remained the same, compared to the concentrated feed used for comparison.

In the Kiel test the quality of the milk, particularly its odor and taste, were checked several times. It was found that, when 3 kg mustard seed groats were fed over a long period of time, no harmful effect on the odor and taste of the milk were observed.

Summarizing it can be said on the basis of the identical results of all three tests that extracted mustard seed groats, both those steamed for two hours and the briefly steamed product, in the quality supplied for the tests,

can be considered a high-grade, high-protein and well-tolerated concentrated dairy cattle feed, which can be fed without hesitation in daily portions of up to 1.5 kg without any harmful effects. It is advisable, however, to mix it with other feeds and to feed it dry.

Translated by Carl Demrick Associates, Inc./IE/db

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Effect of tincture of iodine and mustard oil on the gas metabolism and the blood circulation of the skin.

By Dr. Ernst Sametinger.

(With 7 figures) (Received on 10/27/1930).

While numerous observations are available on the anatomical changes which appear after a local inflammatory irritation (1), the chemical processes in the irritated tissue are less known. But both their quality and their quantity have gained particular interest since it was found that a different pathological tissue, the carcinoma tissue, differs quantitatively and qualitatively in its metabolism from the normal tissue. In addition there is the practical consideration that, on the one hand, the production of local irritations and inflammations serves many therapeutic purposes, and that, on the other hand, the irritated and inflamed tissue itself is the object of therapy, without the chemical side of this changed state being sufficiently known, however.

The present tests will help to clarify this question. The chemical processes were measured by the gas metabolism (oxygen consumption and carbon dioxide release). On the live animal this was only possible by measuring simultaneously the blood circulation of the organ to be examined. This organ was the ear of the dog. As irritants were used tincture of iodine and mustard oil, and for comparison tincture capsici, turpentine oil and xylene.

The gas metabolism after local irritation has already been investigated by Gessler (2), by Croll and coworkers (3) and by Bricker and coworkers (4). Gessler (2) produced a local necrosis by (subcutaneous) injection of 0.2 - 0.3 cc mustard oil or formic acid under the abdominal skin of the pig and excised from the hyperemic areola of the necrotized region after 18-24 hours a piece of skin with the adhering subcutaneous connective tissue. The oxygen consumption of this excised skin piece, which was measured with the method by Barcroft and Warburg, was increased up to 70%, while that of the necrotized tissue was reduced. Groll (3) investigated with the same test arrangement

the oxygen consumption of excised mouse ears after they had beensubjected to the action of different inflammation-causing substances. After brushing on croton oil, theoxygen consumption of the mouse ear increased in the first hour, to drop in the course of a slowly onsetting necrosis within 24 hours to 0. After brushing on weak concentrations of diphenylarsine chloride, the oxygen consumption was increased, after stronger concentrations it was reduced. A low-alcohol cantharides-extract increased theoxidation of the isolated mouse ear only slightly. On the rabbit, Bricker (4) compared the carbon dioxide binding power of the venous blood of a healthy and of an inflamed ear. After the burning or action of croton oil, the carbon dioxide binding power remains unchanged. In other tests Bricker compared the content of oxygen and carbon dioxide in the venous blood of a normal and of an inflamed rabbit ear and tried to draw conclusions therefrom upon the size of the gas metabolism in the inflamed tissue. But the results are not clear.

Methodology.

First preliminary tests were made on rabbits, which were unsuitable, however, because it was not possible to keep the animals under narcosis at a constant body temperature during a 6 hour test. Besides, the ears of rabbits serve to regulate heat and show therefore a greatly varying blood circulation. Furthermore, the total amount of blood is too small for continuous blood sampling for gas analysis. The tests were therefore continued on dogs of 15-40 kg weight, particularly since the measurement of the blood circulation in the ear vein with the thermoelectric watch is only possible in large animals.

The measurement of the entire blood circulation of the ear required at first an orientation about the course of the ear veins of the dog (fig. 1). To this end we had to locate first the point where the main ear vein opens into the vena maxillaris interna. An incision extending caudally from the angle of the jaw parallel to the longitudinal axis of the neck exposed the vena maxillaris interna. The vena auricularis posterior extends from the groove between the easily palpable cross extension of the atlas and the point where the ear starts, and opens from the rear into the vena maxillaris in-

terna. The latter was tied off cranially from the junction of the ear vein and also all its branches up to the vena maxillaris externa. Consequently there was only veinous ear blood up to the confluence of the vena maxillaris externa and the vena maxillaris interna to the vena jugularis externa. Then the vena maxillaris externa was exposed over a distance of 3-5 cm, its trunk tied off, and its branches shut off. For taking blood, a very wide quarts canule was pushed through the stub of the vena maxillaris externa into the vena jugularis externa, overcoming in most cases a veinous valve. (fig. 1).

The blood circulation of the ear was measured either surgically according to the method by Barcroft and Brodie (5) or non-surgically with the thermoelectric watch by Rein (6). The surgical measurement was made by pinching off the vena jugularis externa for the duration of a blood sampling shortly after the confluence of vena maxillaris externa and interna, so that the entire veinous ear blood now flowed from the vena maxillaris interna through the quartz canule into a graduated pipette of 10 cc connected by a rubber tube. A blood volume of 5-10 cc was measured with the stop watch. In case or irregular inflow of the blood, the measurement of the blood circulation was repeated.

Fig. 1. Anatomy of the veins of the dog ear.

In the non-surgical measurements with the thermoelectric watch the diathermy thermocouple of 1.2-2.0 mm diameter was applied in all tests to the vena maxillaris interna. The thermoelectric recording of the blood circulation was effected with a Zeiss loop galvanometer. The thermoelectric watch was calibrated surgically after each test on the same animal with the same position of the couple and with the same heating intensity as in the test itself.

The gas metabolism of the ear results from the size of the blood circulation and from the differences of the content of oxygen and carbon dioxide in the arterial and venous blood. In the determination of the blood circulation according to Barcroft and Brodie, we used for the gas analysis the blood which had flown from the quartz canule into the graduated pipette. In the determin-

ation of the blood circulation with the thermoelectric watch, a partial amount of the blood coming from the ear over the vena maxillaris externa was sucked off through the lateral vena maxillaris externa with a paraffined air-free syringe of 10 cc. The latter method avoids the temporary pinching off of the vena maxillaris externa and preserves thus better the natural circulation conditions. It permits to take only a part of the blood passing by, so that the metabolism of the organ can be measured over a longer time (about 1 minute). The arterial blood was taken from the arteria saphena.

5.0 cc each of the arterial and of the venous blood were transferred to small receptacles which were filled with 0.5 cc 4% sodium fluoride solution and with mercury. The details of the filling and storage by means of this method were described by Janssen (7). The blood was kept in the refrigerator for not more than 2 hours at -3.0 deg.C until the blood gas analysis was made. Then 1.0 cc specimens were tested for oxygen and carbon dioxide according to the combined method by van Slyke.

The difficulties of an exact determination of the gas metabolism of the organ from the blood circulation and the gas content of the blood is due to the fact that the circulation changes occasionally in the organ during the blood sampling, an error which must reappear in the calculated values of the metabolism of the organ. These errors were carefully examined by checking both the inflow of the blood into the graduated pipette and the draining of the blood into the syringe as well as the undisturbed flow before and after the blood sampling by continuous recording with the thermoelectric watch. Thus it was found that the normal conditions for the blood circulation of the ear are disturbed least when the graduated pipette, are entered in table 1. The maximum values were +25 and -42.3% (average 18%) of the blood circulation which existed before and after the blood sampling in the ear. The deviations of the blood sampling with the syringe and the measurement of the blood circulation with the thermoelectric watch showed maximum values of +61 and -24.3% (average 8.6%, see table 1, p.5).

The error, which is caused in the calculation of the gas metabolism of the ear by the change in the blood circulation during the blood sampling, has been

taken into account in the following figures. I calculated the gas metabolism of the ear from both values of the blood circulation, the normal and the disturbed, and from the gas content of the arterial and venous blood, and represented it in the form of triangles on the bars of the oxygen consumption and carbon dioxide release. The error margin thus calculated is small compared to the changes in the gas metabolism by tincture of iodine and mustard oil.

The long duration of the tests averaging 10 hours requires careful treatment of the animals. The best of the various anaesthetics used was numal, because of it slong homogeneous effect up to 24 hours, since it hardly changes body temperature and blood pressure. Each dog received an intravenous injection of 0.5-0.75 cc per kg. During the test the animals were covered down to the endof the neck with a box which was kept at a constant temperature of 28 deg.C by carbon filament lamps. They were protected by a wool blanket against the radiant heat.

Table 1.

Variations of the velocity of flow by sampling with the syringe and with the pipette resp. Error in % of the undisturbed flow existing before the blood sampling, where + means acceleration and - means slowdown.

Blood circulation before sampling	Blood circulation during sampling with the syringe		with the pipette	
cc/min.	cc/min.	error + %	cc/min.	error + %
30	30	0	37.5	+ 25
30	29	- 3.3	37.5	+ 25
26	23	- 11.5	15.0	- 42.3
53	53	0	52	- 2
53	54	+ 2	32	- 39.6
29	29	0	19	- 37.6
57	50	- 12.3	57.7	+ 1.2
57	57	0	57	0
44	40	- 9	40	- 9
32	30	- 6.2	30	- 6.2

Table 1 continued.

50	50	0	56.6	+ 13.2
43	43	0	43	0
37	28	- 24.3	32	- 13.5
36	30	- 16.6	32	- 11
16.5	17	+ 3	14	- 15.1
18	29	+ 61	18	0
22	22	0	14	- 36.3
14	15	+ 7.1	15	+ 7.1

Following the sampling of the arterial blood, the rectal temperature and the blood pressure were measured (with a Hg-manometer on the arteria saphena).

Results

I. Continuous recording of the blood circulation without gas-analytical investigations.

The curves of the blood circulation obtained by recording it continuously for 6 hours with the thermoelectric watch show an almost uniform picture (see fig. 2).

In 8 normal tests, the blood circulation was 20-30 cc per minute. It varies within moderate limits and drops slightly in the course of 6 hours. This behavior is probably due to changes in the systemic circulation, because blood pressure and rectal temperature rise slightly with fading narcosis, accompanied by a vaso-constriction, which also takes place in the skin.

Fig. 2. blood circulation in the dog ear in cc per minute
--- = normal (3 tests). ____ = after brushing with mustard oil (S) (5 tests).

Fig. 3. Blood circulation in the dog ear in cc per minute.
--- = normal, ___ = after brushing with tincture of iodine (J) (3 tests).

Compared to the normal blood circulation, there is at first a drop of the circulation, which lasts only a few minutes, after brushing the outer surface of the ear with various local irritants, which is followed for about 1 hour by

an acceleration and then over several hours by a slowdown of the blood circulation. The initial drop in the blood circulation after the injection of the irritant is a reflex. In the case of mustard oil it must be considered a pain reflex, because it is accompanied by a rise in blood pressure and deep breathing. In the case of the other irritants, the cold produced by evaporation plays a role. The following acceleration of the blood circulation is not a reflex and depends on the chemical changes in the tissue, as it will be shown in later tests. Partly a certain parallelism with the carbon dioxide release of the irritated tissue was found. In a part of the tests this otherwise clear reaction of the vessels is missing, through the reflectory excitation was pronounced enough, namely in the tests where the normal blood circulation of the ear was already high and could probably not be increased anymore. In the further course, the blood circulation drops to or below the normal value, without the chemical reactions of the irritated tissue having achieved again their normal state.

2. Investigation of the gas metabolism before and after the inflammation.

The normal ear of the dog does not have a uniform gas metabolism within a period of 6 hours (see fig. 5). The oxygen absorption during this time is rather constant and shows at most an increase of 50%. But the values of the carbon dioxide release change to a greater extent. If we put the carbon dioxide absorption in relation to the oxygen release in the sense of the respiratory quotient, we obtain in the normal state only once $CO_2/O_2 = 1$. Mostly the values of the r. qu. higher than 1.0 being preferred. In rare cases the r. qu. was 2.0, in one case 0.5. These fluctuations are not due to faulty circulation or congestion of the blood, because they also take place in tests where the blood circulation was constantly checked with the thermolelectric watch and was found to be unchanged.

Fig. 4. Blood circulation in the dog ear after brushing with xylene (X), turpentine oil (T) and tinctura capsici (C) (one test each).

Fig. 5. The normal gas metabolism of the dog ear in the course of 6 hours. black square - oxygen absorption; white square - carbon dioxide release;

Bldr.-blood pressure; Dbl.-blood circulation. Rt - rectal temperature (2 tests).

The displacements in the gas metabolism after mustard oil and also after tincture of iodine are greater than the normal fluctuations. The observation of the blood circulation of the ear under the influence of the locally irritating substances, apart from the brief pain reflex, shows two stages: one stage with increased blood circulation in the first hour and a second stage after the first hour, where the increased blood circulation diminishes again. The blood samplings, which were to provide information about the extent of the gas metabolism was first determined.

The application of mustard oil (see fig. 6) causes in three tests immediately after the brushing a decrease of the oxygen consumption to 1/2 - 1/3. Only in one test did the oxygen consumption remain unchanged. Always the release of carbon dioxide is increased at the same time to the 2-5 fold amount. In the second stage, after 3-4 hours, there is an increased oxygen absorption, however, and a reduced delivery of carbon dioxide, which drops frequently below the normal values. Mustard oil thus causes in the dog ear great changes of the gas metabolism, oxygen consumption and carbon dioxide release showing the opposite behavior.

Tincture of iodine changes the gas metabolism in a different way. The oxygen consumption is increased in the first 6 hours, and if the release of carbon dioxide should be decreased slightly occasionally, the oxygen consumption increases at the same time. The release of carbon dioxide corresponds approximately to the oxygen consumption.

Since it can be concluded from the behavior of the oxygen consumption and of the carbon dioxide release that both qualitatively and quantitatively different metabolic processes appear after the application of mustard oil or of tuncture iodine, it was interesting to see how the metabolism of the tissue would behave in a combination of both effects. I therefore produced first a tincture of iodine effect on a dog ear and then applied mustard oil on the same ear. The characteristic action of tincture of iodine, namely an increase

of the normal oxidations to twice the normal value, is prevented by the following mustard oil actinn. The carbon dioxide production increases in the manner typical of mustard oil.

If the effects of both substances, tincture of iodine and mustard oil, were qualitatively identical, an summation of the effects would have had to take place. But since the gas metabolism after tincture of iodine can be completely reversed by mustard oil, this proves likewise a different action of both substances on the tissue cells.

I have made the following observation on the relation between the gas metabolism of the ear, its blood circulation, and the externally visible changes: The first blood sampling was made 7-30 minutes after the irritants were brushed on. Externally only a reddening of the ear without a roughly measurable increase in thickness was seen at this time. Mustard oil had an accelerating effect, but tincture of iodine caused no substantial changes in the blood circulation. During the second blood sampling, the externally visible hyperemia has mostly completely disappeared, the velocity of flow of the blood had dropped to or below the normal value. The ear brushed with mustard oil has assumed twice the original thickness, a tight endema had formed. After the application of tincture of iodine, no increase in thickness could be found externally after 3-6 hours. During this time the ears were examined several times histologically. They showed all the signs of an inflammation, as it was also described by Fritzler (8) on the guinea pig skin; after tincture of iodine, light edema and slight cell proliferation, after mustard oil greater edematous changes and abundant cell profileration. At the time of the first sampling I examined the ears under the microscope, since Fritzler observed the first morphological changes only after one hour.

I now come to the summary of my observations. Tincture of iodine increases the oxygen absorption alone or the oxygen absorption and the carbon dioxde release at the same time and to the same extent, during an observation period of 6 hours. The skin is extremely red on the outside, but the amount of blood flowing through the ear is only slightly accelerated. Edematization is minor

during the 6 hour-observation period.

Mustard oil reduces the oxygen absorption by 30-50% while increasing at the same the carbon dioxide release considerably within the first hour. In this time the blood circulation is increased, the skin reddened, but no edema is observed yet. In the course of an additional 2 - 5 hours, the oxygen absorption increases above the normal values, while at the same time the carbon dioxide release diminishes considerably. In this condition there is a strong edema, and the blood circulation has dropped to or below the normal value. The effect of both substances was reversible in my tests, it disappeared after a few days. In a comparison of the findings by Gessler, Groll and myself, there is agreement insofar as the oxygen consumption is increased a few hours after the inflammatory irritation in the tissue. Since cell proliferation takes place in this stage already, it follows that the cell proliferation in the inflamed tissue is accompanied by a higher oxygen consumption and differs thus qualitatively from the tumor tissue which lives according to Warburg primarily at the expense of fermentation processes without oxygen consumption. The continuous investigation of oxygen absorption and carbon dioxide release shows the increase of the oxygen consumption as a result of preceding chemical processes where the oxygen absorption is limited, while the carbon dioxide release is considerably increased. Whether organic acids play a role as products of damaged cells with impaired oxidation can not be seen from my tests and requires further study.

The above described processes of the tissue gas metabolism apply only to the action of mustard oil. The effect of tincture of iodine is different and consists in gradual uniform increase of the oxygen absorption and carbon dioxide release. Whether this different behavior is qualitatively or only quantitatively different, can only be decided by a finer analysis of the metabolic processes.

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Translated by Carl Demrick Associates, Inc./IE/db

From: Folia Endocrinologica Japonica. vol. 14, no. 5, p. 31-33.

On the changes in breathing and gas metabolism in serum anaphylactic shock and in shock produced by some capillary toxins.

III. Report on the influence of capillary toxins (croton oil and mustard oil) and parasympathicus-stimulating drugs (pilocarpine and acetyl choline.)

By Dr. R. Kitamura.

(from the I.Med. Clinic of the Imperial University of Kyoto, Japan, Director Prof. Dr. K. Tsuji).

The author had already reported on the causes of capillary permeability increase, reduced gas metabolism and reduction of the total carbon dioxide of the arterial blood in anaphylatic shock, as well as in peptone- and histamine shock. In order to separate the condition after a capillary permeability increase from other shock symptoms, the author tried in this paper to study the influence of the pure capillary toxins, like croton- and mustard oil, on respiration and gas metabolism. At the same time the effect of pilocarpine and acetylcholine on respiration and gas-metabolism was also studied. As test animals were always used female rabbits. Croton oil and mustard oil were used asn an emulsion, adding 1 or 2 drops to a 1 cc thin gum arabic solution. In order to excluse the strictly mechanical effects of the oil emulsion, the influence of the olive oil emulsion was investigated in a control test,which was produced in the same manner as the croton- and mustard oil emulsion. The results were as follows:

1). In rabbits which had been injected with 0.2 - 0.3 cc croton oil emulsion, faster breathing, reduction of the gas metabolism, a slight increase of respiratory quotient, a slight reduction of the total carbon dioxide- and oxygen content of the arterial blood and an increase of the hemoglobin content were observed.

The foregoing changes are on the whole similar to those observed in histamine shock.

2). In rabbits which were injected with 0.25 - 0.45 cc mustard oil emulsion intravenously we observed faster breathing, pronounced shallow breathing, re-

duced gas metabolism, reduction of the respiratory quotient and reduction of the oxygen content of the arterial blood. Despite the last mentioned oxygen reduction, an increased hemoglobin concentration was observed in the arterial blood.
3) In simple injections of olive oil emulsion, practically no changes in respiration, gas metabolism, blood gas and hemoglobin content were found.

On the basis of his test series I-III, the author came to the conclusion that faster breathing, reduced gas metabolism and oxygen and total carbon dioxide content of the arterial blood in the anaphylactic as well as in peptone-and histamine shock are at least partly due to an increase in the capillary permeability, recognizable by the hemoglobin concentration in the arterial blood.
4) In rabbits which had been injected intravenously with 2.0 - 3.0 mg pilocarpine, dispnea, a slight reduction of the oxygen consumption, a marked increase of the respiratory quotient, a drop of the total carbon dioxide content and an increase of the absolute oxygen content were observed. The increase of the oxygen content was less pronounced, compared to the increase of the oxygen capacity, which was probably increased by the hemoglobin concentration.
5) In the rabbits which had been injected intravenously with 0.3 - 0.4 mg acetylcholine, we found dyspnea and faster breathing resp., a slight decrease of the oxygen consumption also an increase of the respiratory quotient and a slight reduction of the total carbon dioxide content of the arterial blood, furthermore a reduction of the hemoglobin-and oxygen content. The respiration was at first shallow, but not considerably. The reduction of the oxygen content was much more pronounced than had to be expected from the simultaneously observed decrease of the hemoglobin content.

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On the effect of irritants on the ascorbic acid content of the adrenal cortex.

By H. Haas

With 3 figures in the text.

(Received on September 3,1957).

Irritants cause, as known, a direct alteration of the tissue which is produced directly by the acting agent. In this "primary effect", they differ, as it was shown by Heubner, in a characteristic manner by their elective influence on individual defined cell elements and by their points of action resp. on several distinguishable tissue constituents. But this does not mean that all the local phenomena must be considered as a direct result of the primarily acting substances, unless certain inflammatory agents, like the bacteria, contain in exceptional cases factors which can be directly coresponsible for the reactive cellular action (Meier). As a rule the primary irritation will release endogenous mechanisms so that secondary biochemical processes will appear in between which enter into the local process and which have the effect that substantially uniform end conditions result despite the initially different mechanism of action and despite the different types of action.

The cation of the irritants is not confined to the local process with sufficiently intensive irritation. As a rule it will rather effect the total organism since the local process as a part of the whole, causes certain regulation-mechanisms in the organism. This becomes obvious by a succession of accompanying general reactions, like the displacement of the blood count with an increase of the total number of leukocytes. These processes were interpreted in the sense of Hoff's concept of a reversal of the vegetative regulations. At present the entire non-specific symptomatiology, which usually appears in acute and chronic damages, under physical as well as under emotional stress, is considered an adaptation syndrome and a result of the activation of the hypophysis-adrenal cortex system (HACS). Undoubtedly the course of a local process which is released by irritants is subject to the same regulation mechanism, provided the irritatio

is sufficiently strong.

Corresponding experiences with the ascorbic acid test, which is considered specific, are available for histamine, where a reduction of the ascorbic acid content was found by Sayers and Sayers, Teppermann and coworkers, Blanchard and Dearborn, Nasmyth, Halpern and Benos, as well as Fortier and Guillemin. It is also known that formalin causes this effect (Avon and coworkers, Domenjoz, Moersdorf, Casentini, Ohi). The same proof was also submitted for dextrane (Domenjoz). Finally Bohnstedt and Lanz observed that turpentine oil irritates the adrenal cortex in rats and guinea pigs and causes a progressive transformation of its structure which takes place over the hypophysis in the opinion of the researchers.

All these findings extend almost exclusively to the reaction of normal animals, but not of animals which have been subjected to hypophysectomy. Only for histamine have Blanchard and coworkers described that the extirpation of the hypophysis has no effect on the ascorbic acid content of the adrenal cortex. But it is difficult to decide whether the intact organism reacts to the entire group of irritants without exception in a different manner than the hypophysectomized animal and to what extent the interaction between local process and total organism is maintained after the removal of the hypophysis.

In the analysis of the biochemical causes and consequences of irritation effects one can not be satisfied, however, with the finding that in these processes the independence of the parts in the sense of the whole is always limited and that the rule applies here too: the part reacts, but the whole regulates. This is nothing special according to biological laws; we can therefore hardly expect a principally different course of the process in the specific case of the irritants.

It is much more difficult to find a valid answer for a different problem complex, since this range is less accessible to experimentation. Despite many efforts it is still not clear whether and to what extent the entire local and general reaction course can be interpreted as a result of the primarily acting substance and to what extent it is caused indirectly by a non-specific

irritant in the tissue by mechanisms formed endogenously in the center. Furthermore it is questionable if all irritants show principally the same behavior in their consequences or whether there are among them substances with differentiated mechanisms, as it was found by Meier and coworkers for croton oil and turpentine oil, which hold a special position among the irritants. It is also unknown to a great extent how the signalling mode takes place by which originally localized process becomes a matter of the organism.

It seemed therefore logical to include these questions too in the present investigations and to test them experimentally within the framework of the given methodological possibilities which are determined by the characterization of the influence of irritants on the HACS.

Methods

The determination of the ascorbic acid content of the adrenal glands was made on rats according to the method by Roe and Kuether. All determinations were made on the hypophysectomized rats at the same time, namely 72 hours after completion of the operation. In the aniamals whose n. ischiadicus had been severed, the time interval between the operation and the determination of the ascorbic acid was 7 days, since preliminary tests had shown that the ether narcosis and the severing of the nerves cause a brief minor depletion, which disappears again after a few days. In order to be quite safe, the ascorbic acid test was made uniformly on the 7th day after the operation.

The irritants were generally injected into the foot soles of the rats. The amount of liquid was always 0.1 cc. Specifically the following substances were used: croton oil 2% dissolved in peanut oil; turpentine oil 40% dissolved in peanut oil; dextrane 6%; egg albumin undiluted; formalin 0.75%, histamine 1:1000; NaCl 0.9%.

Some additional tests were made with croton oil and mustard oil with epicutaneous application of the irritants. The rats received 3 drops each of the undiluted irritants on the shaved back.

In the preliminary treatment of the animals with antihistamines, sympathicolytics, ganglia blockers etc., these substances were always supplied s.c.

in milligram per kg, immediately before the application of the irritants into the foot soles.

Specifically the following substances were used: hydrocortones (hydrocortisone) phenobarbital (5-ethyl-5-phenyl-barbituric acid); regitin (2-(N'-(p-tolyl-N'-m'oxyphenyl)-imidazoline) soventol (N-phenyl-N-benzyl-4-amino-1-methyl piperidine), acetopromazine ((N'(3'-dimethylamino)-propyl-3-acetophenothiazine) and pendiomide (N,N,N',N'-3-pentamethyl-N,N'-diethyl-3-azapentylene-1,5-diammonium dibromide).

The usual techniques were used for counting the leucoctyes and eosinophiles. The variations of the blood count were calculated in percent of the starting value. The foot thickness was measured with a gauge directly below the ankle joint. The swelling appearing under the influence of the irritants is indicated in fig. 1-3 regularly in % of the norm. All data relate to mean values on 6-12 animals.

The effect of the irritants on the foreign body-granuloma was investigated likewise on rats according to the data by Meier and coworkers. The irritants were added to the crude cotton tampon in decreasing dilution series.

The weight of the granulomas was determined uniformly in all test series on the 7th day after the s.c. implantation. The weight difference, resulting from the comparison with the salt-saturated compacts on the opposite side, is entered in table 4 as a percentage value. 4 animals were used for each test.

Test results

A. The effect on leucocytes, eosinophiles, condition of swelling and granulation growth.

All irritants effect a local swelling in the rat after subplanter injection, which develops as a rule rather rapidly and has exceeded its maximum at the latest in the course of 4 hours in all selected substances.

Fig. 1. Normal rats: subplantar application

._____. swelling on foot; x- - -x leucocytes; o -.- o eosinophiles.

The changes in the white blood count consist within the first hour of the

action of the irritants in a slight decrease of the leucocytes, followed by a granulocytosis, which was not very pronounced with histamine, formalin and turpentine under the selected test conditions, similar to granulocytopenia. At the same time an eosinophile drop sets in which is uniform with all irritants and shows only minor differences in its chronological course. As known, the eosinophile test can not be evaluated in its results without skepsis for technical and statistical reasons. In connection with other changes in the blood count, this factor can not be overlooked completely, however, particularly since the uniformity of the phenomenon is noted as a positive feature with all irritants. The uniform result of all test reactions means that all these phenomena are to a great extent of a non-specific nature and are therefore unsuitable for a characterization of the individual irritants. They are in addition identical with the blood count changes observed by Hoff in the vegetative total reversal and are in agreement with the cellular transformation of the blood composition described by Selye within the framework of the adaptation syndrome.

After the hypophysectomy, the leucocytosis disappears again with all irritants. The eosinophile reaction likewise fails to appear as a rule. For turpentine oil and croton oil there is regularly a drop below the starting values in all animals under these circumstances, to an extent which can be statistically ascertained (fig. 2).

The acute local reaction phase, measured on the swelling of the feet, however, is practically identical in the normal and in the hypophysectomized animals, with the exception of turpentine oil. The local process is thus positively not inhibited by the hypophysectomy, in contrast to the sequence of accompanying general syndromes. The relation between the local and the general irritation reaction, which is found regularly under normal conditions with insufficient irritation intensity, is thus eliminated to a great extent by the removal of the hypophysis. In this result we can see an indirect confirmation of the view by Hoff who attributes a determinant role to the nervous disturbance in the leucocytic response to the irritation. It seems therefore understandable that there is no movement of leucocytes after a lesion of the hypotha-

lamic core regions, which is unavoidable in a hypophysectomy.

The unchanged course of the acute swelling processes at the site of the irritation effect does not mean, however, that the local state of irritation can act unhindered in its subsequent phenomena after removal of the hypophysis, for example, in the proliferation phase of the mesenchymal tissue parts. On the basis of this consideration, we made some additional orientative tests with foreign body granuloma test, with which Meier and coworkers had already investigated a number of irritants and with which they had proven a clear granuloma enhancing effect for croton oil from the group of irritants discussed in our tests.

Table 1. Foreign body-granuloma. Weight change in %.

Concentration	Normal rats					Hypophysectomized rats				
	1	2	3	4	5	1	2	3	4	5
1:2.5		+88					+ 43			
1:5		+67								
0	+206	+46			+ 4	+146	+ 18			+ 3
1:100	+108	+19	+156			+ 94	- 4	+ 78		
1:1000	+ 68		+147	+ 9		+ 55		+ 57	+ 9	
1:10000	+ 25		+ 38			+ 11		+ 14		
1:1000000			+ 16							

1 - croton oil; 2 - turpentine oil; 3 - mustard oil; 4 - histamine; 5 dextrane.

In this method, the substances to be tested are introduced into a cotton tampon and they acted this way over several days. Our own experiences are limited toa period of 7 days, after which the weight of the granulomas was determined. Naturally a relatively complex process can only be determined summarily in such a test, where in addition to the reaction of the connective tissue, leucocytes, fibrocytes and vascular processes are involved, but which promises in its totality relatively useful information about the reactivity of the granuloma tissue. The weight difference of the implantation granulomas

charged with physiological salt solution and these saturated with irritants provide thus in connection with the hypophysectomy the possibility of determining to what extent the individual irritants influence the total functions of the granuloma tissue, to what extent they behave differently in normal animals, and how the removal of the hypophysis can affect these processes.

Fig. 2. Hypophysectomized rats: subplantar application.
.-. swelling on foot; x--x leucoctyes; o-.-o eosinophiles.

For these determination we selected specifically croton oil, mustard oil, turpentine oil, dextrane and histamine, and calculated the measured deviations of the granuloma weight from the norm in percent. The indicated values represent mean values from the results on four granulomas each.

In addition to the granuloma-enhancing effect of croton oil already found by Meier on normal rats, a similar effect could be demonstrated for mustard oil. In addition, turpentine oil, which is likewise a specially irritating substance according to Meier, also increases to a minor extent the growth of the granulomas, while dextrane and histamine have no effect in this respect. This different behavior of the various irritants in the normal animals is not surprising, however, since it is already known from the studies by Meier and coworkers that the irritants display different effects in this test. This finding is expanded insofar by our own observations as only the three substances mustard oil, turpentine oil and croton oil, which enhance the reaction of the mesenchyma in the normal animals, show the same effect after a hypophysectomy. The special position of these substances in the hypophysectomized animals is thus emphasiced not only by the behavior of the eosinophiles, but also by the remaining granuloma-enhancing effect.

B. Effect of the ascorbic acid content of the adrenal gland.

All selected irritants cause in the normal rat a decrease of the ascorbic acid content of the adrenal gland. With croton oil, the effect on the adrenal gland sets in very rapidly, so that the depletion effect can already be demon-

strated very clearly ½ hour after the injection into the foot sole. When the oil is dropped on the back sin, it takes somewhat longer, as expected, until the same result can be determined to a significant extent. After an action of 2 hours, the ascorbic acid content has clearly dropped below the norm, even in this application. The same effect has mustard oil in epicutaneous application. An action of 1-1½ hours suffices already with this irritant to reduce the ascorbic acid content considerably. Egg albumin and dextrane lead with intraplantar supply in the course of 60 minutes to a clear reduction of the ascorbic acid content in the adrenal gland. Turpentine oil and histamine have the same effect after staying for 2 hours in the organism, and with formalin an effect can be determined positively only after 3 hours. A control test with common salt, carried out in the same manner, leaves the ascorbic acid content of the adrenal gland unchanged.

After the hypophysectomy, the effect of egg albumin, dextrane, formalin and histamine on the adrenal glands can no longer be determined at a time when these irritants reduce the ascorbic acid content in normal animals, while croton oil and mustard oil definitely reduce the ascorbic acid content during the same period, as in normal animals. The extent of the reaction also remains about the same if we consider that the rats still had a slightly higher ascorbic acid content in the adrenal gland than normal animals 72 hours after the hypophysectomy. In the case of turpentine oil, a decrease of the ascorbic acid content in the adrenal gland could be observed clearly only 3 hours after the supply. But a decreasing tendency can already be found after 2 hours, while egg albumin, dextrane, formalin and histamine leave the ascorbic acid values unchanged at this time, so that not the slightest deviation from the normal ranges can be found even in a statistical examination.

The conspicuous result of this test series is thus that only the irritants which enhance the growth of granulomas on the hypophysectomized animals and cause a decrease of the eosinophiles in the blood under the same conditions show a clear depletion effect after removal of the same organ, while all other chemical irritants do not show these active components in all tests. It must

be assumed that some irritants have somehow an effect on the adrenal gland, independent of the hypophysis. It consists probably in a direct influence on this organ, unless we want to conclude from thes findings upon the presence of extra-hypophysial mechanisms with adrenotropic functions. But this is a presently unproven hypothesis. It is a fact, however,that all tested irritants act on the adrenal glands in the presence of the hypophysis over the latter and cause changes there which are similar to those appearing after the administration of adrenocorticotropic hormone. The biological significance of this effect is still unclear. Obviously this reaction of the HACS does not activate the inhibiting substance of the inflammatory reaction cortisone and cortisol, since the acute local reaction phase, measured on the swelling of the foot, is the same after subplantar injection of the irritants in normal and in hypophysectomized animals. The proliferation of the mesenchyma after removal of the hypophysis with the irritants which enhance this reaction is likewise not inhibited, as the findings with croton oil and mustard oil in the foreign body-granuloma test show. One is therefore inclined to assume that the cooperation of the adrenal gland rather serves to enhance and stimulate the profileration processes in the supporting connective tissue than to inhibit or eliminate them. In support of this theory it can be said that Tomani could achieve an enhancing effect on the local irritation processes with aldosterone in the case of turpentine oil. In addition, aldosterone and desoxycorticosterone enhance the growth of the foreign body-granuloma according to Desauleles and Meier. As known, the adrenal cortex does not havethe same variety of steroids and partly different effects as they are produced by other animals. Despite this exceptional position, it still seems to contain enough prerequisites to adapt its output to the respective needs.

C. Effect of irritants on pretreated animals.

If it is true that only a aprt of the irritation phenomena in the range of the primary action area can be considered as a direct consequence of the substances applied, and all other processes are secondarily dependent on the

Table 2. Ascorbic acid content of the adrenal cortex in mg/100 g body weight (mean values + standard deviation).

Normal rats.

Irritants	form of administration	1	P	2	P	3	P	4	P	5	P
croton oil	i.plant	342 ± 40 (4)	0.30	155 ± 10 (5)	0.001	170 ± 19 (4)	0.001	152 ± 14 (12)	0.001	63 ± 23 (3)	0.001
	epicut					228 ± 17 (3)	0.05	96 ± 16 (4)	0.001		
mustard oil	epicut			195 ± 20 (4)	<0.01			121 ± 24 (7)	0.001		
turpentile oil	i. plant.					242 ± 14 (7)	0.05	213 ± 14 (8)	0.001	118 ± 24 (5)	0.001
histamine	i. plant.					234 ± 20 (3)	< 0.1	228 ± 12 (23)	0.001	127 ± 23 (5)	0.001
egg white	i. plant.					155 ± 23 (5)	0.001	169 ± 20 (10)	0.001	195 ± 47 (5)	< 0.02
dextrane	i. plant					194 ± 16 (5)	0.001	165 ± 16 (10)	0.001	92 ± 14 (4)	0.001
formalin	i. plant.							261 ± 15 (9)	0.1	186 ± 24 (9)	0.001
NaCl						351 ± 29 (4)	< 0.2	275 ± 24 (4)	0.1		

The bracketed numbers refer to the number of test animals. Normal value of the untreated animals 303 ± 37 (26).

1 - after 15 min.; 2 - after 30 min.; 3 -after 60 min.; 4 - after 120 min.; 5 - after 180 min.

Table 3. Ascorbic acid content of the adrenal cortex in mg/100 g body weight.

Hypophysectomized rats.

Irritants	form of administration	1	P	2	P	3	P
croton oil	i. plant	272 ± 30 (3)	<0.01	285 ± 11 (13)	0.001	230 ± 16 (4)	< 0.01
mustard oil	epicut.	128 ± 35 (4)	0.001				
turpentine	i. plant			305 ± 11 (10)	< 0.005	225 ± 31 (4)	< 0.01
histamine	i. plant			351 ± 21 (6)	0.30		
egg albumin	i. plant			360 ± 15 (8)	0.04		
dextrane	i. plant			365 ± 18 (8)	0.40		
formalin	i. plant			380 ± 43 (8)	>0.40		

The bracketed numbers refer to the number of test animals. Normal value of the hypophysectomized animals 379 ± 59 (18).

1 - after 60'; 2- after 120'; 3 - after 180'.

Fig. 3. Normal rats: croton oil: subplantar application, preliminary application s.c. simultaneously. (a - untreated animals); b - croton oil after treatment with...)

.-. swelling, foot; x---x leucocytes; o-.-o eosinophiles.

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action and formation of chemical intermediate links, we will have to ask ourselves, as far as the order of the accompanying general systems is concerned, particularly the activation of the adrenal glands, to what extent these changes are caused by a direct influence of the irritants or whether they are caused only by secondary mechanisms. This also means that we have to ask for the signalling mode by which the localized process causes the regu-

lation by the organism. This passage of the irritation process from the source proper to the entire organism can take place principally in two ways: the nerval or the humoral way. In the latter case an outpour of adrenalin or the release of histamine could be causally the indirectly releasing factors. In addition, a drop of the adrenal cortex hormone level, due to increased peripheral consumption, must be considered. This problem must be tackled experimentally by a preliminary treatment of the animals with antagonistic substances or by a preliminary administration of adrenal cortex hormone, so that it is possible to check the dependence of the irritants on such an intermediate process.

Soventol, acetopromazine, regitin and pendiomide inhibit the local irritation process, if they are supplied in small doses simultaneously with the subplantar application of croton oil, the extent of the swelling being more or less reduced, depending on the type of preliminary treatment. This result is in agreement with the findings of other investigators who have described similar effects (Kramer, Morrison, Briot, Halpern, Eichenberger, Meier, Gross, Kitchin, Wilhelmi, Theobald, Domenjoz, Jacques, Leger, Brown, Clark, Marquardt). Phenobarbital in narcotic doses and hydrocortisone weaken the local process. Concurrently the leucocytosis fails to appear in all test series, and the result is generally an increased and prolonged leucoplenic phase in which we can see probably a sign of reduced adaptation. Only after acetopromazine and regitin is the drop of the leucocytes not increased; the secondary rise likewise remains at least approximately determinable within the test period. All substances which cause an inhibition of the local irritation process are thus capable of weakening or eliminating certain general syndromes.

Unaffected, however remain the drop of eosinophiles and of ascorbic acid, which appear in the treated animals to the same extent as in normal rats. We have to keep in mind, however that, apart from pendiomide and regitin, all other substances used for the preliminary treatment affect the ascorbic acid content of the adrenal glands. The addition of croton oil, however, effects an additional drop of the ascorbic acid content so that a significant differ-

Table 4. Ascorbic acid of the adrenal cortex in mg. 00 g body weight.

Preliminary treatment	1		2	3		4		5
Hydrocortisone	200.0	252 ± 20 (3)	0.2	croton oil	152 ± 14 (12)	Hydrocortisone	166 ± 17 (4)	0.5
phenobarbital	100.0	202 ± 36 (4)	<0.02	croton oil		Luminal	158 ± 14 (4)	>0.9
regitin	1.0	354 ± 12 (3)	0.2	croton oil		Regitin	142 ± 54 (4)	>0.9
soventol	1.0	251 ± 36	0.1	croton oil		Soventol	168 ± 13	>0.9
acetopromazine	2.0	103 ± 10 (4)	0.001					
pendiomide	2.0	280 ± 28 (7)	0.4	croton oil		Pendiomide	96 ± 17 (4)	<0.02
regitin	1.0	354 ± 12 (3)	0.1	Dextrane	165 ± 16 (10)	Regitin	206 ± 30 (6)	0.2
soventol	1.0	251 ± 36 (7)	0.1	Dextrane		Soventol	188 ± 14 (4)	>0.5
pendiomide	2.0	280 ± 28 (7)	0.4	Dextrane		Pendiomide	117 ± 10 (4)	0.1
regitin	1.0	354 ± 12 (3)	0.2	egg albumin	169 ± 20 (10)	Regitin	117 ± 16 (6)	< 0.1
soventol	1.0	251 ± 36 (7)	0.1	egg albumin		soventol	156 ± 32 (3)	>0.9
pendiomide	2.0	280 ± 28 (7)	0.4	egg albumin		Pendiomide	158 ± 32 (3)	<0.9
regitin	1.0	354 ± 12 (3)	0.2	histamine	228 ± 12 (23)	Regitin	165 ± 20 (3)	0.1
soventol	1.0	251 ± 36 (7)	0.1	histamine		Soventol	260 ± 35 (3)	0.4
pendiomide	2.0	280 ± 28 (7)	0.4	histamine		Pendiomide	132 ± 44 (3)	0.02

1 - dose mg/kg, s.c.; 2 - P norm/preliminary treatment; 3 - irritants: untreated animals; 4 - irritants after; 5 - P irritant/preliminary treatment + irritant.

The bracketed numbers refer to the number of test animals.

Table 5. Ascorbic acid content of the adrenal cortex in mg/kg body weight.

1	2	3	4	5
croton oil	152 ± 14 (12)	240 ± 18 (11)	0.01	0.001
egg albumin	169 ± 20 (10)	241 ± 14 (6)	<0.05	0.02
dextrane	165 ± 16 (10)	249 ± 31 (7)	<0.1	<0.05
histamine	228 ± 12 (23)	231 ± 21 (4)	0.05	>0.09

1 - prior to plantar injection; 2 - normal animals; 3 - after severing the n. ischiadicus; 4 - P1 norm/irritant + ischias op.; 5 - P2 irritant/irritant plus ischias op.

The bracketed numbers refer to the number of test animals.

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ence results in each case. In agreement with these results, Birggs and Munson found that pentobarbital can not prevent the depletion effect of histamine. Nor can chloropromazine weaken the eosinopenia caused by ACTH and formalin resp. (Kresbach and Sailer).

The depletion effect of croton oil in subplantar administration can be prevented exclusively after severing the n. ischiadicus. At the same time the leucocytosis fails to appear. The local process is also less pronounced, as it can be seen from the reduced swelling. The eosinophile drop remains, however, unchanged, even after the nervous sensible elements are interrupted (see fig. 3). There are probably highly differentiated methods of action and independent mediators which bring about an influence on the eosinophiles and the ascorbic acid content in the adrenal glands.

The elimination of the depletion effect after severing the ischiadicus is found for dextrane and egg albumin in normal rats in the same manner as for croton oil. The sensible elements extending in the ischiadicus must therefore have a particular significance. In the case of histamine, the influence on the adrenal glands obviously takes place in completely different ways, because only the antihistamine soventol is here prophylactically effective. But the

selection of the antihistamine used seems to play a role here. A positive antagonism with elimination of the drop of the ascorbic acid content in the adrenal glands, caused by histamine, was achieved with phernergan (Fortier) as well as pyribenzamine and phenoxadrine (Tepperman). With benadryl and neoantergan the histamine effect can not be stopped (Nasmyth). Chloropromazine, which reduces the ascorbic acid content of the adrenal glands, inhibits the histamine effect only in large doses (Nasmyth).

In the hypophysectomized animal, croton oil can reduce the ascorbic acid content in the adrenal gland, after the ischiadicus has been severed, as it can be seen from a comparison of the two test series with and without supply of croton oil, where the nerve had been severed 7 days prior to the determination of the ascorbic acid and the hypophysis had been removed 72 hours before.

Discussion of the test results.

It is difficult to give a simple explanation for the widespread and complicated effect of irritants, or to derive a systematology of the various types of action from the presented material. The selection of substances is too small for this purpose. Besides, their manner of reaction in the acute phase can only be summarily determined with the measurement of the condition of swelling. Likewise the nerval regulation processes and the consequences for the tissues in the course of the reparative phase were considered exclusively from the viewpoint of blood count changes and the influence on the HACS. For these resons the phenomena are represented less in their variety than in their common traits, particularly since the various irritation variants lead to similar effects, despite their differences. Even at the site of their action, the specificity of their type of action is limited, because the detailed mechanisms take place in the complex system of a cellular unit. The latter is capable of responding to irritations, of reacting. Consequently the local irritation processes do not stop even in their initial phases at their first contact with the tissues at the influence on a single point of action. They are rather involved in the common functions of the tissues and in the metabolism of the tissues. We obtain thus as a rule a resultant from a number of factors which

can be considered in their nature as primary or secondary and which were in their totality a relatively uniform influence on the structures and functions in the range of their origin.

Table 6. Ascorbic acid content of the adrenal cortex in mg/100 body weight.

after severing ischiadicus seven days before	+ hypophysectomy 72 hours before
without addition of irritant	275 ± 21 (8)
after croton oil	263 + 22 (7).

The bracketed numbers refer to the number of test animals.

This local process can be observed as a total complex by measuring the swelling in its chronological course without specifying the scale of the partial phenomena. But it can be expected that the effects observed in this test are substantially the same for all irritants. This local process is not inhibited to any considerable extent by the hypophysectomy in any of the test animals. The course of the local tissue reactions thus does not seem to be recurrently subject to a regulation of the internal secretion on the part of the HACS or to be decisively controlled by the latter.

Not all irritants display a granuloma-enhancing effect after prolonged action, as it is exerted by croton oil, mustard oil and turpentine oil. In this respect there are thus clear differences in the various irritants. As far as they have an effect on the growth of the granuloma, the latter remains even after removal of the hypophysis. But it is greatly limited in its extent under these conditions. We must therefore expect both direct and indirect action components by which the proliferation processes are more enhanced in the presence of the hypophysis. In favor of an intervention of humoral factors it can be argued that aldosterone increases the course of the inflammation in the turpentine oil abscess and enhances the development of the granuloma tissue in the foreign body-granuloma test. Furthermore it should be kept in mind that only those irritants cause a connective tissue reaction which show a depletion effect even on the hypophysectomized animal, while all other chem-

ical irritations do not show any of these action components under the same test conditions.

The irritation process, which is primarily a local process, is at the same time subject to the regulation of the total organism, as shown by the accompanying general syndromes in the form of a change of the blood count, the drop of the eosinophiles, and the decrease of the ascorbic acid values in the adrenal glands. All these action components must be considered non-specific effects, since they appear in complete uniformity, independent of the nature of the individual irritant and of the nature of their primary action characteristic. They are not suitable to characterize the type of active substances.

After the hypophysectomy, these phenomena disappear completely with most of the irritants. It can therefore be concluded that these substances act over the hypophysis on the adrenal glands and cause there a similar effect as the adrenocorticotropic hormone. Whether a control of the infallmation process takes place, which can mean inhibition or enhancement, depending on the type of the activated hormone, can not be seen from the findings. For the local reaction phase this process can not be very significant, since the condition of swelling is practically completely identical in normal and in hypophysectomized animals after subplanter injection of the irritants. A clear statement is not possible as to how the influence from the focus on the adrenal gland takes place since the depletion effect can be prevented exclusively by severing the n. ischiadicus. The sensible fibers in the n. ischiadicus thus seem to be of considerable importance. But this does not apply with the same uniformity to the behavior of the eosinophiles, which still drop in the case of croton oil after the severing of the n. ischiadicus. The interaction between the organism and the irritated region obviously takes place in different ways. Only a part of the mediators uses the nerve paths, while other signaling modes are conducted humorally.

Even on the hypophysectomized animal, croton oil, turpentine oil and mustard oil are capable of reducing the ascorbic acid content of the adrenal glands, while all other irritants remain ineffective under these test conditions. It

must therefore be expected that certain irritants can act on the adrenal glands independent of the hypophysis. There are thus among the irritants certain substances with a special type of action which goes beyond the local tissue processes and which still shows the characteristic of the acting agent, even in the various regulation mechanisms of the organism.

Summary

For the analysis of the action of irritants, we investigated their influence on the white corpuscles, the eosionphiles, and the ascorbic acid content of the adrenal glands on the normal and hypophysectomized rats, and in addition measured the course of the swelling processes and the influence on the granuloma tissue.

Croton oil, mustard oil and turpentine oil hold a special position among the irritants because they effect an eosinophile drop and a depletion of the adrenal glands even in the hypophysectomized animal. Besides, croton oil, turpentine oil and mustard oil are the only ones among the selected irritants which effect the growth of the granuloma in the foreign body-granuloma test. Severing the n. ischiadicus can not prevent the reduction of the ascorbic acid content in the adrenal glands after interplantar injection of the irritants. In the case of histamine, the same effect could be achieved by a preliminary treatment with the antihistamine soventol. It is shown that in some types of irritants even the processes of the reactive regulation mechanism depend indirectly on the nature of the acting agent.

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EXPERIMENTAL STUDY OF LIPAEMIA CLEARING BY LIPOPROTEIN LIPASE AND ALTERED BLOOD COAGULABILITY WITH RESPECT TO FATTY ACID COMPOSITION OF DIETARY TRIGLYCERIDES*

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(Received for publication December 4, 1964)

INTRODUCTION

Lipoprotein lipase catalyzes the hydrolysis of the triglyceride moiety of chylomicrons and lipoproteins and brings about clearing of lipaemia (Korn, 1959). Its specificity of action with respect to fatty acid composition of different dietary fats causing lipaemia has, however, not been studied. Recent studies have further shown that post-prandial lipaemia and blood coagulation are interrelated in some obscure way (Merskey and Nossel, 1957; Greig and Rundle, 1957; Mustard, 1957 and Poole, 1958). In order, therefore, to investigate into these mechanisms, triglycerides of various composition were employed to induce alimentary lipaemia in rabbits and its clearing in vitro by heparin-induced lipoprotein lipase was determined along with blood coagulability changes.

MATERIAL AND METHODS

The studies were conducted on rabbits weighing 1.5 Kg. averagely which were given a stock diet made up of grain-bran mixture and fresh greens. The fats employed were so selected as to represent various degrees of saturation and content of polyethenoid essential fatty acids (iodine value and saponification number determined). These were butter, pure ghee, hydrogenated pea-nut oil (Dalda), mustard, olive and cod-liver oils.

Experiment 1 — Study of lipaemia and lipoprotein lipase activity : Fasting rabbits were gavaged through a No. 5 India rubber catheter

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with 8 ml. of different fats in a single feed. For each fat 6 animals were employed. Simultaneously a parallel control batch of animals was sham-fed with normal saline to eliminate the effect of tube feeding. The experiment was performed in pair-fed animals, one each from the test and control groups. After 3 hours, blood was sampled from the heart in centrifuge tubes containing sodium oxalate, plasma was separated by centrifugation at 2000 rpm. and stored in the referigerator. The extent of lipaemia was determined in a Klett-Summerson photoelectric colorimeter using a 640 m.mu. filter against distilled water blank. The increase in optical density of the lipaemic plasma from the control represented the value of actual lipaemia.

In the study of lipoprotein lipase the substrates differed in each group depending on the type of fat fed but the enzyme source remained constant. To liberate the enzyme in high concentration two normal rabbits were injected subcutaneously with sodium heparin 10 mgm./Kg. body weight and blood was collected after 40 minutes. The plasma was separated by high centrifugation and stored in the cold to preserve the enzyme. The lipaemic plasma samples from each group were then mixed with the post-heparin plasma at room temperature according to the technique of Cairns and Constantinides (1955). The loss in optical density of the mixture was recorded colorimetrically at 20 and 40 minutes and value of lipaemia clearing expressed as % of the original lipaemia determined at the 0 hour.

Experiment II — Effect of lipaemia on blood clotting in vitro : In this experiment the immediate and delayed effects on blood clotting were studied separately after giving single feeds of the different fats to various groups (8 animals in each) as described already. The observations were made at 0 hour, 3 hours and 24 hours of fat feeding and the following parameters were considered, all tests being performed in duplicate :—

(i) Whole blood clotting time by Lee and White method

(ii) Plasma racalcification time (Dacie, 1956)

(iii) Plasma prothrombin time using Geigy's thromboplastin tablets

(iv) Platelet count by Lempert's modification of Kristenson's technique (Dacie, 1956)

(v) Plasma fibrinolysin activity by modified Mole's method (Srivastava et al, 1962).

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RESULTS

Fatty acid composition of triglycerides: Determination of the iodine value of each fat showed that animal fats were highly saturated while vegetable fats and marine oil contained very large quantities of unsaturated fatty acids. A comparison of the vegetable oils showed that hydrogenated pea-nut oil contained much less of the unsaturated fatty acids than its unhydrogenated partners. While considering the content of polyethenoid essential unsaturated fatty acids (Deuel, 1957) it was found that the total percentage of linolenic and/or arachidonic acids (E.F.A.) was comparatively much higher in mustard oil (vegetable) and cod-liver oil (marine), less in olive oil (vegetable) but very low in butter and ghee (animal) and hydrogenated pea-nut oil (vegetable) — (see table I).

Production of lipaemia: Lipaemia was produced by a single dose of the fats employed but it was significantly higher than the control only in case of butter ($P<0.01$). In the case of 'Dalda' the degree of lipaemia was quite marked but variation among the individual animals was so great that the values became insignificant statistically (Table I).

TABLE I.

Effect of fatty acid composition on lipaemia production and its clearing by lipoprotein lipase in vitro

Fat	Iodine value	Percentage of E.F.A. as linoleic, linolenic and/or arachidonic acid	Difference of optical density between lipaemic & control plasma at 0 hour	% lipaemia clearing at 20 min.	% lipaemia clearing at 40 min.
Butter	36.3	4-5	0.230 ($P<0.01$)	6.5	17.7
Pure ghee	30.5	6.2	0.064	9.7	29.1
Hydrogenated pea-nut oil (Dalda)	69.9	4-5	0.139	23.9	46.4
Mustard oil	105.2	25	0.018	71.4	100
Olive oil	91.3	15	0.016	47.7	60.9
Cod-liver oil	95.9	25-26	0.068	19.1	23.8

Lipaemia clearing by lipoprotein lipase: The injection of heparin in normal rabbits appeared to stimulate the liberation of a lipolytic

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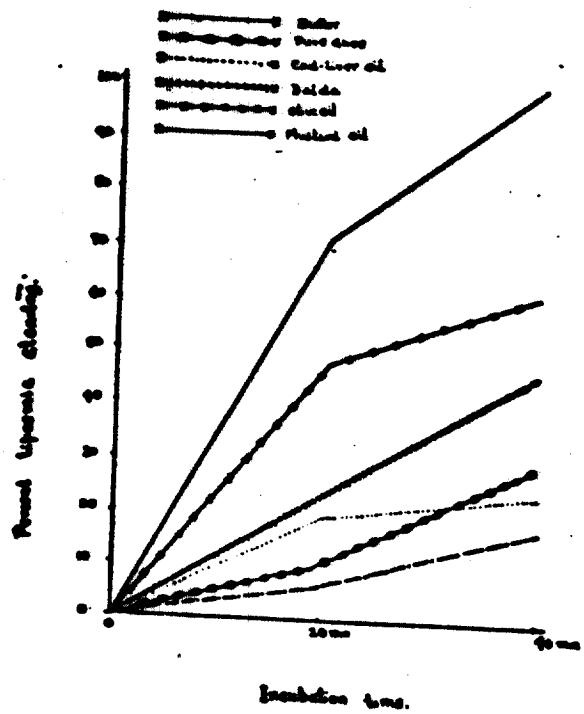
enzyme, lipoprotein lipase which acted on the lipaemic plasma substrates to effect differential clearing of turbidity. In some fat induced lipaemias the clearing was complete or almost complete within 40 minutes while in the case of others it was only partial. In case of butter, ghee and cod-liver oil the clearing activity was very poor while it was very good in mustard oil induced lipaemia. Although there was a fair degree of correlation between the iodine value, content of E.F.A. of triglycerides and lipaemia clearing with most fats employed yet a great discrepancy was noted with cod-liver oil lipaemia (Table I and Fig. 1).



-2. Rate of lipaemia clearing by lipoprotein lipase in case of different triglycerides

Blood coagulation studies : Among the saturated fats pure ghee and hydrogenated pea-nut oil (Dalda) caused significant acceleration of the pre-clotting mechanism of clot formation ($P<0.05$). Butter, however, produced less marked similar changes ($P \sim 0.1$). None of these

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fats exerted a significant effect on clot lysis. The unsaturated lipids, mustard, olive and cod-liver oils had no appreciable effect on the blood clotting mechanism. It was further noted that whatever significant clotting changes occurred were at the 24 hour period and not at the 3 hour period (Figs. 2-6).

DISCUSSION

The present investigations have shown that the structure and composition of dietary triglycerides did determine to some extent the degree of lipid accumulation in the blood, its metabolic clearing by lipoprotein lipase and its effect on blood coagulability. It was found that butter and hydrogenated pea-nut oil (Dalda) which contained a high content of saturated fatty acids and very little essential unsaturated fatty acids (E.F.A.) could produce a marked lipaemia, but

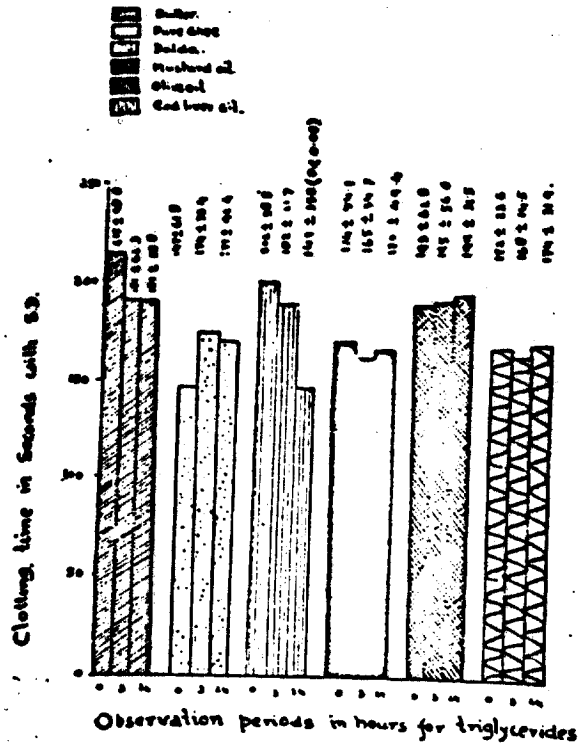

Fig. 2. Whole Blood Clotting Time.

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ghee failed to do so. It may be possible that during the preparation of ghee by boiling milk fat the fatty acids were polymerised and thereby impeded intestinal absorption while butter which besides fatty acids contained protein and water behaved in a different way. The marked difference in the production of lipaemia between animal fats and vegetable/marine fats may be dependent on the differential rate of absorption and/or various rates of metabolic removal from the circulation to the fat depots (Swell et al., 1955 and French et al., 1958).

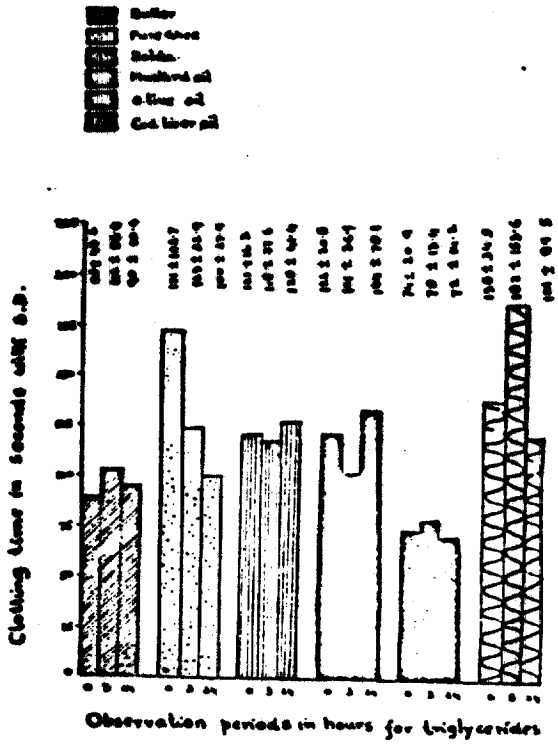

Fig-3 Plasma recalcification time.

It is well known that one of the important mechanisms for the disposal of neutral fats in combination with proteins circulating in the blood as chylomicrons and lipoprotein is through the hydrolytic action of the enzyme, lipoprotein lipase (Engelberg, 1960). The object of the present study was to examine indirectly if fatty acids of different

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structure and chemical composition would facilitate or inhibit the lipolytic action of this enzyme in vitro. It was observed that fats like mustard and olive oil containing large quantities of E.F.A. as linoleic, linolenic and/or arachidonic acids could stimulate the catalytic action of this enzyme to clear the lipaemia quickly. The reverse was true with butter, ghee and hydrogenated pea-nut oil (Dalda) which had very

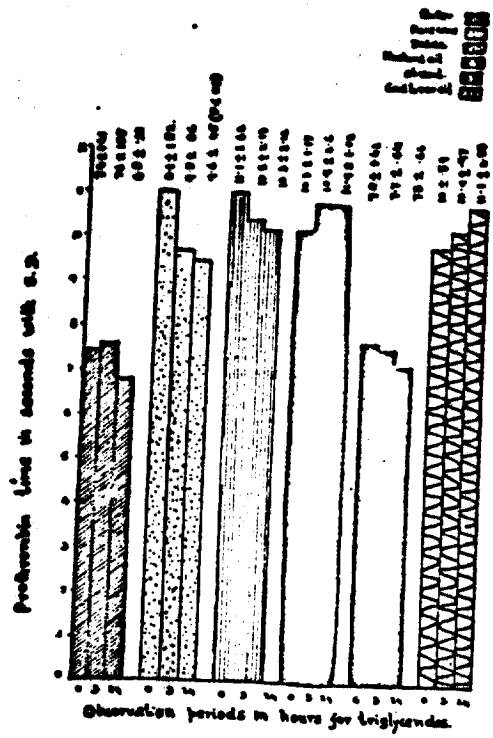

Fig. 4. Plasma prothrombin time.

little E.F.A. facilitating the stagnation of these fats in the blood. Ananthasamy and Cama (1964) also observed that in vivo saturated fats and cholesterol depressed the release of lipoprotein lipase from the heart in rats. As regards cod-liver oil, however, it is difficult to explain its failure to clear lipaemia quickly inspite of the high content of E.F.A. According to Kessler et al. (1964) some yet obscure factors may cause hyperlipaemia by depressing the lipoprotein lipase activity and it is

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possible that during the absorption of cod-liver oil some such factor was released into the blood which exerted an inhibitory action on lipolysis. However, as lipaemia clearing and lipolysis are not synonymous (Nestel, 1964 and Engelberg, 1964) the estimation of products of hydrolysis could have solved this problem. Further work in this direction is, therefore, indicated.

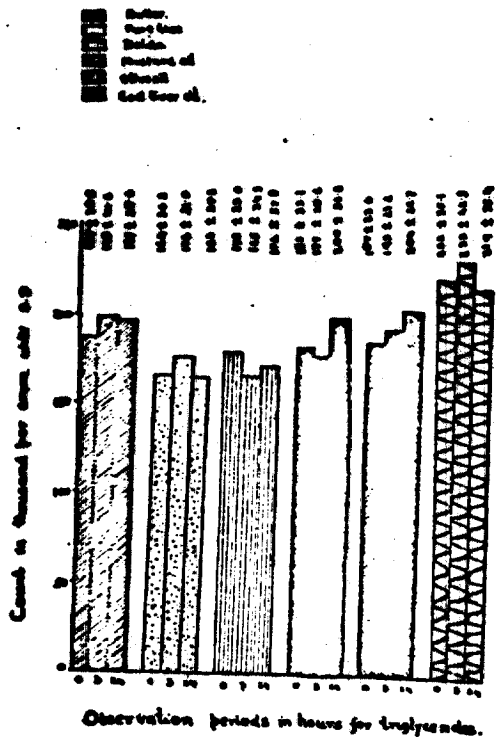

Fig-8. Blood platelet Count.

As far as the blood clotting mechanism was concerned significant acceleration of clot formation in vitro was noted with saturated fats poor in E.F.A. content but no such effect was seen with those rich in E.F.A. In our previous studies also butter feeding to rats caused significant acceleration of the clotting mechanism and precipitated intravascular thrombosis when coupled with other systemic factors (Chakravarti and Zaidi, 1964). For these changes the chylomicrons

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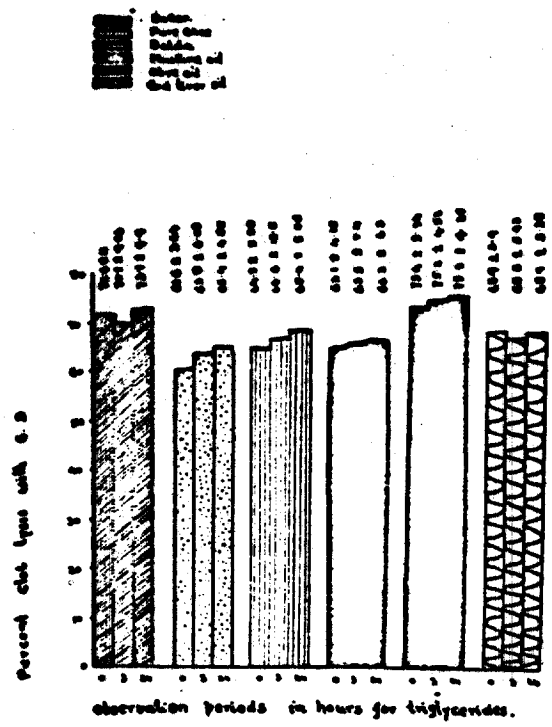

Fig-6. Plasma fibrinolysin activity.

and ß-lipoproteins which are showered into the circulation after taking triglycerides rich in saturated fatty acids may be responsible. Poole (1958) ascribed a platelet like activity to chylomicrons due to their content of ethanolamine phosphatide and Riding and Ellis (1964) observed antiplasma activity of ß-lipoproteins. These findings were interesting and suggested that E.F.A. or some allied substances present in dietary triglycerides may have a protective role against sustained hyperlipaemia and augmented blood coagulation. Further probing into this intricate problem is indicated by employing purified E.F.A. of different types.

SUMMARY

1. Rabbits were fed saturated and unsaturated fats in a single feed and the extent of lipaemia, its clearing by lipoprotein lipase and the state of blood coagulability were studied in vitro.

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2. The saturated fats like butter and hydrogenated pea-nut oil caused marked lipaemia but ghee failed to do so. The unsaturated fats like mustard, olive and cod-liver oil did not produce significant hyperlipaemia.

3. Lipoprotein lipase activity in clearing lipaemia of mustard and olive oil was high but extremely low activity was seen when butter, ghee, 'Dalda', and cod-liver oil were employed.

4. The saturated fats tended to accelerate the blood coagulation mechanism while the unsaturated ones had no such effect.

ACKNOWLEDGEMENT

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PULMONARY EMBOLISM BY POWDERED HOUSEHOLD-MUSTARD INCIDENT TO SELF-INDUCED ABORTION

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Medical literature is replete with various methods and substances used to produce abortion. The following case is unusual and to my knowledge is the first reported instance of the use of ordinary powdered kitchen-mustard as an abortifacient. The embolic complications add a flavorful sidelight and emphasize the size of particle that can be picked up by the uterine veins and produce pulmonary embolism.

CASE REPORT

D. M., a white woman 31 years of age, was admitted to the hospital March 10, 1953, in shock with a blood pressure of 60/40. A history was obtained of vaginal bleeding for 2 days prior to admission. Approximately 1 month prior to admission, she had taken 32 tablets of an unidentified ergot preparation in an attempt to produce abortion; however, this was of no avail. Since that time, she had daily taken hot sitz baths with powdered mustard added to the bath water, without having achieved the desired result. Two days prior to admission, she mixed a 1¼ oz. can of powdered kitchen-mustard in the hottest water she could possibly stand and took a douche. This induced vaginal bleeding and cramps began shortly thereafter. The fetus was passed and disposed of at home.

On admission, approximately 500 ml. of chocolate-colored thick urine was obtained. She was immediately taken to the operating room and under gas-oxygen-ether anesthesia, dilatation and curettage were performed with removal of a large amount of foul smelling, partially necrotic placenta. The uterus was flabby, and bleeding could be controlled only by insertion of a large pack into the uterus and vagina. Blood culture subsequently yielded *Clostridium perfringens*. During the next 2 days she received 6 500-ml. transfusions of O Rh-negative blood with Witebsky substances added. She developed lower nephron nephrosis and hemolytic anemia, and died suddenly 45 hours after admission to the hospital.

AUTOPSY FINDINGS

The body was that of a well-developed, well-nourished white woman measuring 66 inches in length and weighing 128 lb. There was, in addition to an icteric discoloration of the skin and sclerae, a "butterfly" erythematous rash over the bridge of the nose and cheeks. On opening the body cavities, there was approximately 500 ml. of dark brown fluid within the peritoneal cavity, 300 ml. in the

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Dr. Lovitt is Assistant Medical Examiner.

FIG. 1 (upper). A granule of household powdered mustard embedded, sectioned and stained in routine fashion. Hematoxylin and eosin stain. × 140.

FIG. 2 (middle). Obliteration of a uterine vessel by a thrombus composed of a granule of household powdered mustard. Note the inflammatory reaction in and about the vessel wall. Hematoxylin and eosin stain. × 140.

FIG. 3 (lower). Thrombosis of a branch of a pulmonary artery by an organizing exudate about a granule of mustard. Hematoxylin and eosin stain. × 140.

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FIGS. 1–3

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right pleural cavity and 150 ml. in the left pleural cavity. The heart exhibited no gross abnormalities and weighed 380 Gm.; the pulmonary artery contained fluid blood. Together, the lungs weighed 1900 Gm., were soft and boggy in consistency and dark red-purple in color with loss of crepitation in all areas. No areas of pulmonary consolidation were noted. The liver (weight 1825 Gm.) showed a mottled yellow-brown color with accentuation of the lobular pattern. The spleen was enlarged, weighing 380 Gm., and on cut section revealed a firm, adherent, dark red-purple parenchyma. Each kidney was swollen and weighed 180 Gm. On cut section, each revealed an irregular pale swollen cortex with obliteration of strial markings. The medullary portions appeared congested, with accentuation of the striations. Within the renal pelves, there were dark red-brown accumulations of what appeared to be blood clot. Similar material was present within the lumen of the ureters and bladder. The uterus, enlarged to 3 times normal size, revealed a soft, flabby muscle exhibiting a yellow-brown color. Within the uterine cavity, the remaining endometrium presented a green-brown necrotic surface. There was no perforation of the uterine wall. The mucosa of the cervix was hemorrhagic and edematous. Multiple sections of the brain showed no gross abnormalities.

Microscopically, within the veins of the wall of the uterus, there were numerous emboli, obviously composed of plant cells with surrounding early organization and thrombosis of the vessels (Fig. 2). In the adventitia of these vessels, there was an acute inflammatory exudate. Throughout all sections of the lungs, there was marked congestion, and many of the small vessels contained numerous hexagonal plant cells found to be identical with the granules of powdered household-mustard (Figs. 1 and 3). Accompanying these emboli, there was organization of the thrombi obliterating the lumens of the vessels. An active necrotizing inflammation was present within the bronchi, with ulceration of the mucosa and a submucosal infiltration. Throughout all areas of the liver, there was an acute central lobular necrosis. The peripheral areas of the lobules were not affected. In the periportal regions, there were a few acute inflammatory cells. The kidneys presented a typical picture of hemoglobinuric nephrosis and the spleen showed marked congestion but was otherwise not remarkable: The anatomic diagnoses were: Self-induced abortion by a concentrated solution of household mustard with pulmonary embolism caused by mustard granules; central lobular necrosis of the liver and lower nephron nephrosis; septicemia (*Clostridium perfringens*); hemolytic anemia; acute ulcerative bronchitis and bronchopneumonia.

DISCUSSION

It appears evident that this woman sustained embolism as the result of a douche under pressure of a hot concentrated aqueous solution of household mustard. The mustard granules were picked up by the uterine veins and transported to the lungs where they lodged in the small pulmonary vessels. The embolized mustard granules varied between 0.4 and 1 mm. in diameter. In itself the embolus was apparently not massive enough to have caused acute death by mechanical blockage of the pulmonary vessels; instead, death resulted from

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hemolytic anemia, necrosis of the liver and hemoglobinuric nephrosis. Whether the *Cl. septicemia* or mustard produced the hemolytic anemia with its attendant complications remains at this time unanswered.

Mustard in solution releases a volatile oil consisting of allylisothiocyanate (C_3H_5NCS), which is the active irritant.[1] This oil is released by a reaction between the glycoside and an enzyme in the presence of water. Undoubtedly, the abortion can be attributed to the irritant action of the volatile oil of mustard which may also have been instrumental in producing the hemolytic anemia.

SUMMARY

A case of self-induced abortion utilizing a concentrated solution of powdered household mustard is presented. Another bizarre feature of this case is embolism of mustard to the pulmonary vessels. Death occurred as the result of central lobular necrosis of the liver, hemoglobinuric nephrosis and septicemia caused by *Clostridium perfringens*. The exact etiologic relationship between the mustard and the hemolytic anemia is obscure.

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THE RESPONSE OF THE FENESTRATED VESSELS OF THE SMALL INTESTINE OF RATS TO APPLICATION OF MUSTARD OIL

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PLATES VII–XV

IN recent years the morphological changes in small vessels responsible for the great increase in their permeability that occurs after injury have been studied extensively in skin and in muscle, and both the pattern of leaking vessels and the ultrastructural appearances of their walls are known in many different types of injury.

However, the small blood vessels of many organs have a very different structure from those found in skin and muscle. In particular, many organs contain so-called fenestrated capillaries in which multiple pores, often apparently closed by a thin diaphragm, are present in endothelial-cell cytoplasm. Except for the vessel of the renal glomerulus, relatively little attention has been paid to the behaviour of vessels of this type under physiological or pathological conditions.

Most capillaries and small venules in the mucosa of the small intestine are fenestrated. The present paper describes the topography and ultrastructural features of the response of mucosal vessels in the rat small intestine to application of mustard oil.

MATERIALS AND METHODS

Animals. Sixty-five male rats of the Sprague-Dawley strain weighing 150–200 g were used.

Injection into intestinal loops. Animals were anaesthetised with ether. Through a longitudinal abdominal incision a loop of upper small intestine was exposed and ligated with cotton at two points approximately 3 cm apart; care was taken to place the ligatures on either side of a large branch of the mesenteric artery. Mustard oil, 0·20–0·25 ml, was injected with a fine needle into the bowel lumen until the loop was moderately distended. The concentration of mustard oil used was 12 drops in 25 ml olive oil (Florey, 1960). Control animals were given a similar injection of 0·20–0·25 ml of olive oil. The bowel was replaced and the abdomen closed in layers. Loops of bowel from normal unoperated rats given an intravenous injection of carbon and killed 1 hr later were also examined.

For experiments of more than 6 hours' duration the abdomen was reopened 2–3 hr after injection, the ligatures were removed and the wound was reclosed in the usual way.

To *demonstrate permeable vessels* in the bowel a colloidal suspension of carbon black (Günther Wagner, Pelikan Werke, Hanover, Germany, batch C11/1431a) was injected in a dose of 0·1 ml per 100 g body weight into the lateral tail vein at the desired time after injury. After allowing at least 30 min. for clearance of carbon from circulating blood, the

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animal was killed with ether and the injected loop of bowel was removed and fixed in 4 per cent. buffered formaldehyde in saline. After fixation, preparations were dehydrated in ethyl alcohol and cleared in cedarwood oil.

Evaluation of cleared specimens. To gain detail of the distribution of labelled vessels specimens were examined in three ways—some loops were examined intact, others after they had been opened along the anti-mesenteric border and pinned flat on cardboard, and still others after they had been cut into freehand rings 1–2 mm thick with a razor blade. All preparations were examined under a Zeiss dissecting microscope with epi-illumination.

Histology. After clearing, blocks were embedded in paraffin, and sections cut at 6 μm were stained with either haematoxylin and eosin or 1 per cent. toluidine blue.

Electron microscopy. In addition to intravenous injection of carbon suspension, animals from whom specimens were taken for electron microscopy were given an intravenous injection of 25–30 mg of cadmium-free ferritin dissolved in 0·5 ml saline. This was given either concurrently with the carbon suspension or at a later time, as indicated below.

Animals were killed with a blow on the head, and the ligated loop of bowel was removed as rapidly as possible and immersed in 2·5 per cent. glutaraldehyde in cacodylate buffer. After approximately 5 minutes' fixation the loop was opened and examined with a dissecting microscope. At this stage areas containing blackened vessels are clearly visible. Suitably labelled areas were selected, excised with a fresh razor blade, cut into small pieces, fixed for a further 1 hr in glutaraldehyde, post-fixed in osmium tetroxide and embedded in Araldite as described by Ham and Hurley (1965).

From each block preliminary sections 1 μm thick were cut, stained with toluidine blue and examined by light microscopy. Intramural and intraluminal deposits of carbon are clearly visible in such sections and it is possible to determine the position within the injured bowel occupied by individual vessels seen with the electron microscope.

For electron microscopy, sections were cut on a Huxley ultramicrotome with glass knives, mounted unsupported on copper grids, doubly stained with uranyl nitrate and lead citrate and examined in either a Siemens Elmiskop I or a Hitachi 11a at 60 kV.

Many sections from multiple blocks from several animals were examined at each of the times described below.

Drugs. Histamine acid phosphate was obtained from B.D.H., London. Lymph-node permeability factor was given to us by Professor D. A. Willoughby.

RESULTS

Time-sequence and pattern of vascular labelling in mustard oil-induced injury to small bowel

In normal rats given an injection of carbon and killed 1 hr later there is dense blackening of large venules within Peyer's patches (fig. 1). In addition small deposits of carbon are present within very occasional villi. No labelling is visible in either muscularis or serosa.

In control animals given an injection of olive oil the mucosal labelling is similar to that of normal rats, but widespread labelling of venules is present in both muscularis and serosa. This labelling is maximal in animals given carbon immediately after operation and decreases progressively to disappear after about 2 hr. Beneath ligature sites the labelling is more intense, involves both capillaries and venules, and is present at all times examined in both test and control animals.

Cleared specimens were examined from animals given an injection of carbon immediately (ten rats), 1–2 hr (six rats), 4 hr (eight rats), 6 hr (five rats) and 24–48 hr (six rats) after injection of mustard oil.

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Animals given carbon immediately after injury show uniform mucosal labelling throughout the injected loop (fig. 2). The labelling involves capillaries and small venules deep to the epithelium covering the villi, and also both venules and capillaries surrounding the gland crypts (fig. 3). Labelling in the muscularis and serosa is confined to venules and is similar in pattern to, but more intense than, that seen in corresponding control loops.

The mucosal labelling is unaltered 1 hr and 2 hr after mustard oil injection, is similar in pattern but decreased in intensity at 4 hr, and has almost disappeared by 6 hr. Labelling of muscularis and serosa decreases progressively to disappear about 4 hr after injection of mustard oil.

Twenty-four to 48 hr after injury no labelling is visible in mucosa, muscularis or serosa except in Peyer's patches and immediately deep to ligature sites.

Light-microscope appearances

At the concentration used mustard oil excites an acute inflammatory reaction in mucosa and submucosa but does not cause necrosis or ulceration of small-bowel epithelium, which is stimulated by the injury to produce large volumes of mucus, which distend the injured loop of bowel. Mucosal oedema is visible within 1 hr of injection and emigration of polymorphs into the injured area begins shortly afterwards.

The distribution of carbon within the damaged area is similar to that seen in cleared specimens. In histological sections the carbon deposits in venules are intramural, but definite localisation of the carbon present in relation to capillaries is often not possible. In 1-μm sections some capillary deposits are clearly intramural.

Three-fold increase in the concentration of mustard oil causes a more severe inflammation with a similar pattern of mucosal labelling in the first 2–3 hr. However, epithelial necrosis and widespread mucosal ulceration are visible 6–8 hr after injury and perforation of the injected loop occurs in some rats.

Electron-microscope appearances of mucosal blood vessels in small bowel damaged by mustard oil

Specimens were examined from normal and control animals, and at 45 min., and 2, 4, 24 and 48 hr after injection of mustard oil. Animals killed at 45 min. were given carbon and ferritin immediately after injury. Control and normal animals, 2-hr, 4-hr and some 24-hr animals were given carbon and ferritin 30–40 min. before they were killed: other 24-hr and the 48-hr animals were given carbon 2 hr after injury and ferritin 10 min. before they were killed.

Mucosal vessels in control and normal animals appear similar. Some capillaries and venules are lined by continuous endothelium, but a variable proportion of the circumference of most capillaries and small venules is much thinner and contains fenestrae 0·1 μm or less in diameter. In favourable sections diaphragms are visible across the fenestrae. The number of fenestrae in individual vessels varies considerably. Most fenestrae lie on the side of the vessel adjacent to the epithelium of villus or gland crypt. Intercellular junctions

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appear similar to those of vessels in skin or muscle. They are rarely seen in fenestrated areas and in capillaries are commonly on the opposite side from the fenestrations. Both fenestrated and non-fenestrated vessels have a continuous basement-membrane.

Ferritin is present in high concentration in vascular lumina, and scattered particles are visible in extravascular tissues with no apparent increase in concentration adjacent to either fenestrae or intercellular junctions. Occasional carbon particles are present within vessels, but none is visible in vessel walls or extravascular tissues.

In the muscularis and serosa of control animals given an injection of olive oil, intramural marker particles are present in some venules. The endothelium of these vessels is of normal appearance and gaps are present in some of them. No carbon deposits in relation to capillaries are present in muscularis or serosa.

The dense carbon labelling in Peyer's patches is seen in 1-μm sections to be in the walls of the post-capillary venules. These vessels (figs. 4–6) are lined by a high cuboidal or columnar type of endothelium whose cytoplasm contains very few pinocytic vesicles, moderate numbers of other organelles and many granules of RNA, mostly in small scattered rosettes. Lymphocytes in large numbers are present at all stages of passage through the venular wall. Many particles of carbon and ferritin are visible between adjacent endothelial cells, adjacent to emigrating lymphocytes and against the basement-membrane at the periphery of the venule. Carbon and ferritin are not seen within the cytoplasm of either lymphocytes or endothelial cells.

Forty-five minutes after mustard oil injection the endothelium of most vessels in the mucosa appears normal except for occasional cells that exhibit a minor degree of damage of the type to be described below. Deposits of carbon and ferritin lie deep to the endothelium of many capillaries and venules (figs. 7–12). In such deposits the carbon particles lie between endothelium and apparently unaltered basement-membrane, but most of the ferritin spreads through the basement-membrane and particles of ferritin are visible in high concentration in extravascular tissues.

Gaps are present in the endothelium covering some intramural deposits of marker particles in both capillaries and venules (figs. 10 and 12), and in many other labelled vessels closed intercellular junctions overlie the intramural carbon and ferritin (figs. 7–9). Large gaps are present in some of the vessels whose endothelium shows obvious damage (fig. 11). Intramural carbon deposits are not visible in fenestrated areas of endothelium and no increased concentration of extravascular ferritin is apparent adjacent to fenestrae.

In muscularis and serosa intramural marker particles are present in some venules of normal appearance. The appearances are similar to those in control animals.

Two hours after injection of mustard oil many vessels in the injured mucosa appear normal, but others are clearly abnormal. In these vessels the endothelial cells appear swollen, their cytoplasm contains large vacuoles and the number of pinocytic vesicles is much reduced. Fenestrae are unaltered in

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FENESTRATED INTESTINAL VESSELS AND MUSTARD OIL

FIG. 1.—Mucosal aspects of small bowel of normal rat given an injection of carbon and killed 1 hr later. Large venules within Peyer's patch show dense blackening. Surrounding mucosa is unlabelled. ×c. 3.

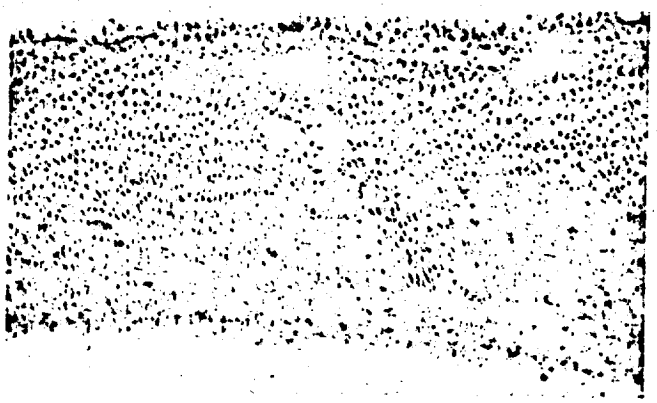

FIG. 2.—Mucosal aspect of cleared specimen of small bowel of rat given carbon immediately after mustard oil injection. Small deposits of carbon are visible in most villi. ×c. 3.

FIG. 3.—Freehand slice of cleared specimen of small bowel of rat given carbon immediately after mustard oil injection. Fine carbon deposits are visible in small vessels beneath epithelium covering the villi. The vessels surrounding mucosal glands are more heavily labelled. Vessels in muscularis are not labelled. ×180.

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FIG. 4.—Electron micrograph (EM) of post-capillary venule in Peyer's patch of normal rat given an injection of carbon 1 hr before killing. Several lymphocytes (L) are present between tall columnar endothelial cells. Carbon (↗) is visible at base of endothelium. ×5000.

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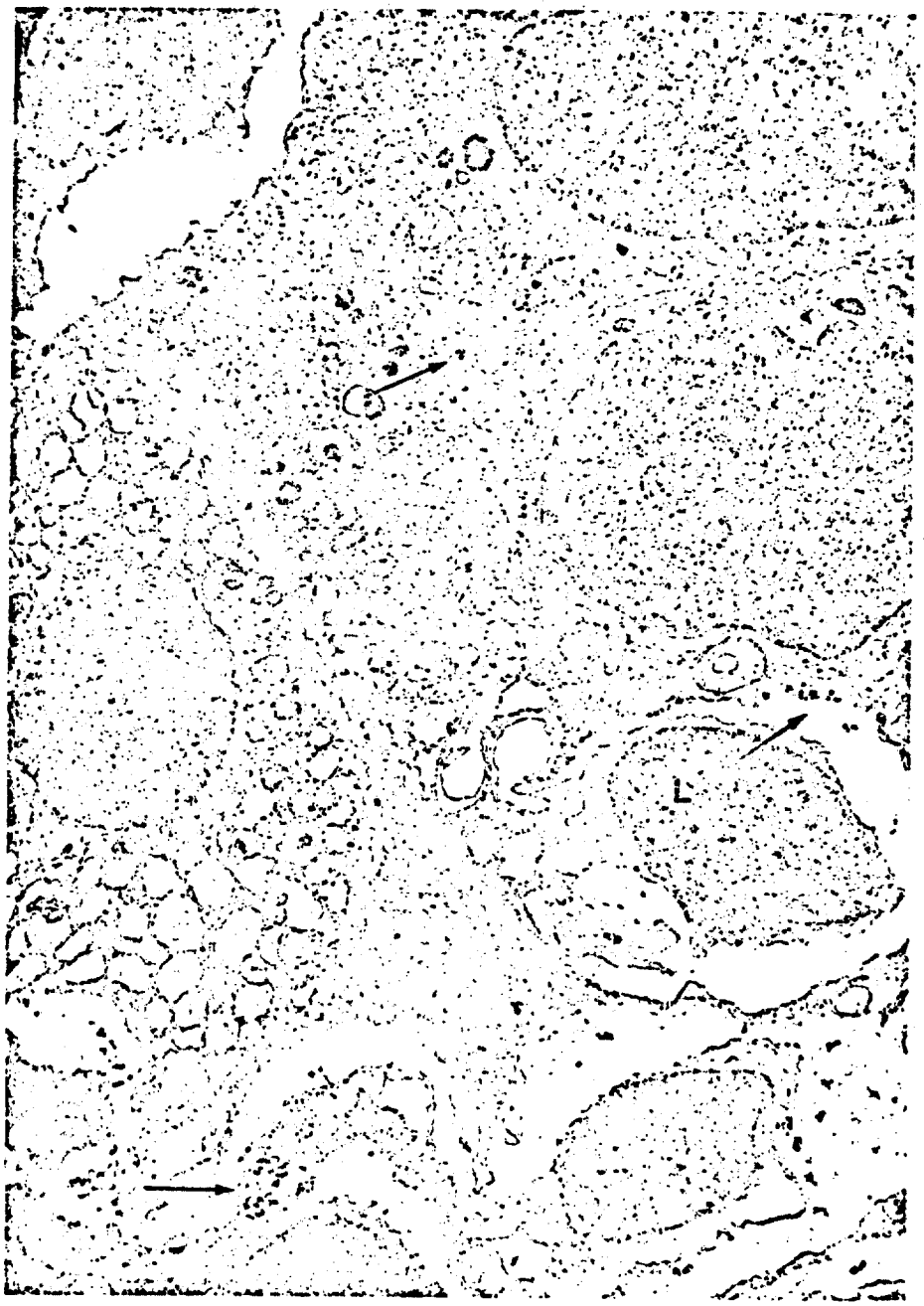

FIG. 5.—EM. Portion of wall of post-capillary venule in Peyer's patch of normal rat given injection of carbon 1 hr before killing. Two lymphocytes (L) are present deep to columnar endothelial cells. Carbon is visible between endothelial cells (↗) and also deep to endothelium (↗). × 10,900.

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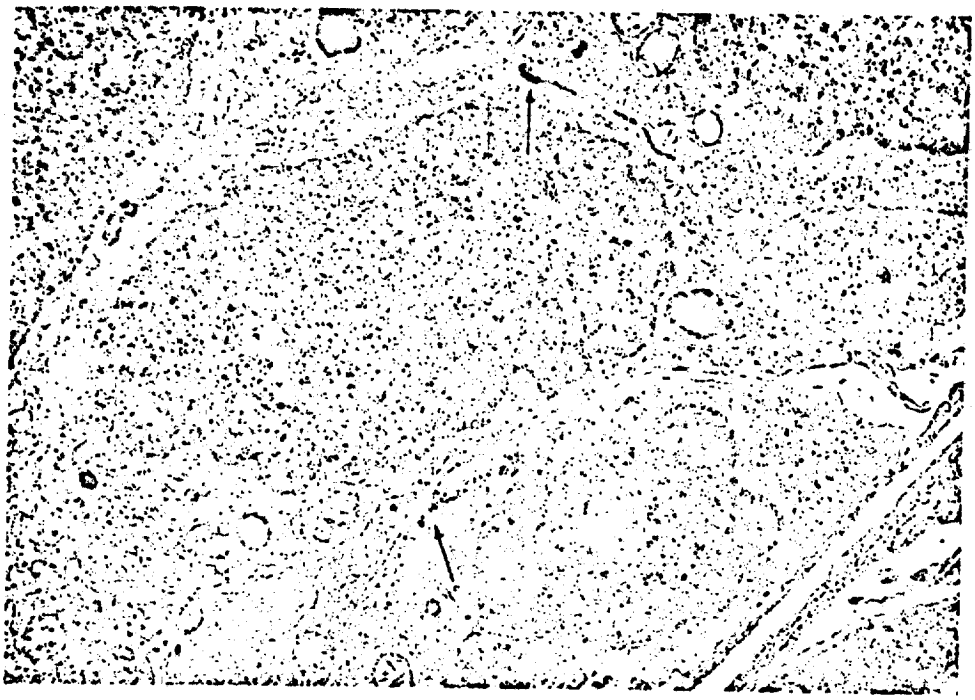

FIG. 6.—EM. Part of wall of post-capillary venule in Peyer's patch of normal rat given injection of carbon 1 hr before killing. Cytoplasm of endothelial cells contains mitochondria, endoplasmic reticulum, many small rosettes of RNA and large lipid droplets. No pinocytic vesicles are visible. Carbon particles (↗) lie in spaces between endothelial cells. ×10,000.

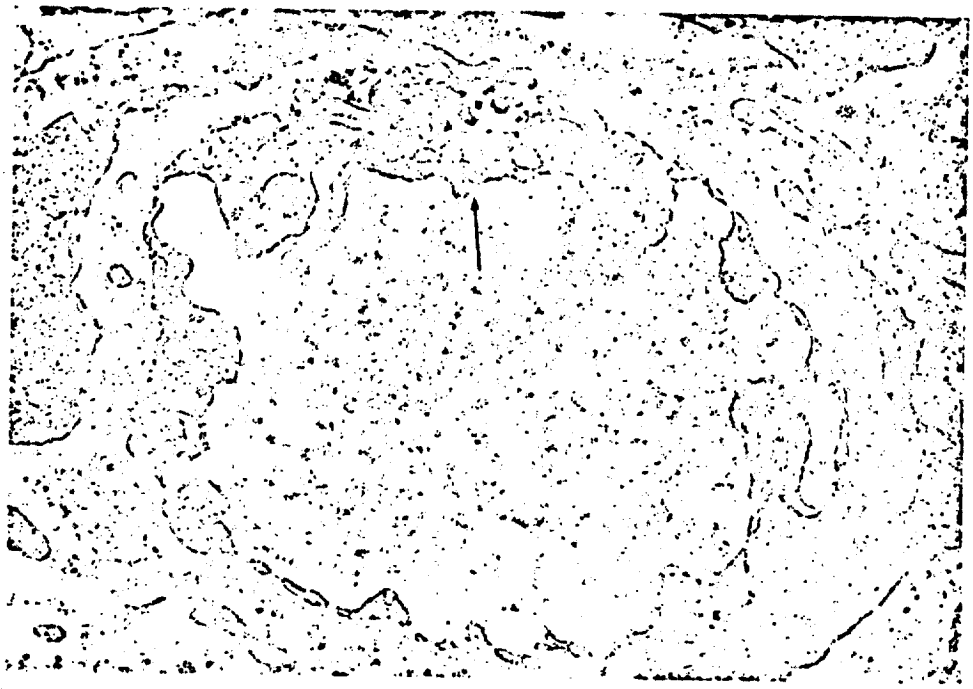

FIG. 7.—EM. Capillary in mucosa of small bowel of rat given carbon immediately after injection of mustard oil and killed 45 min. later. A large intramural deposit of carbon lies beneath the closed intercellular junction (↗). Many fenestrae are present on opposite side of capillary, whose endothelium looks normal. ×22,500.

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FIG. 8.—EM. Capillary in small-bowel mucosa of rat given carbon immediately after injection of mustard oil and killed 45 min. later. A large intramural carbon deposit lies close to closed intercellular junction (↗). Several fenestrae are present in upper part of capillary, whose endothelium appears normal. ×19,200.

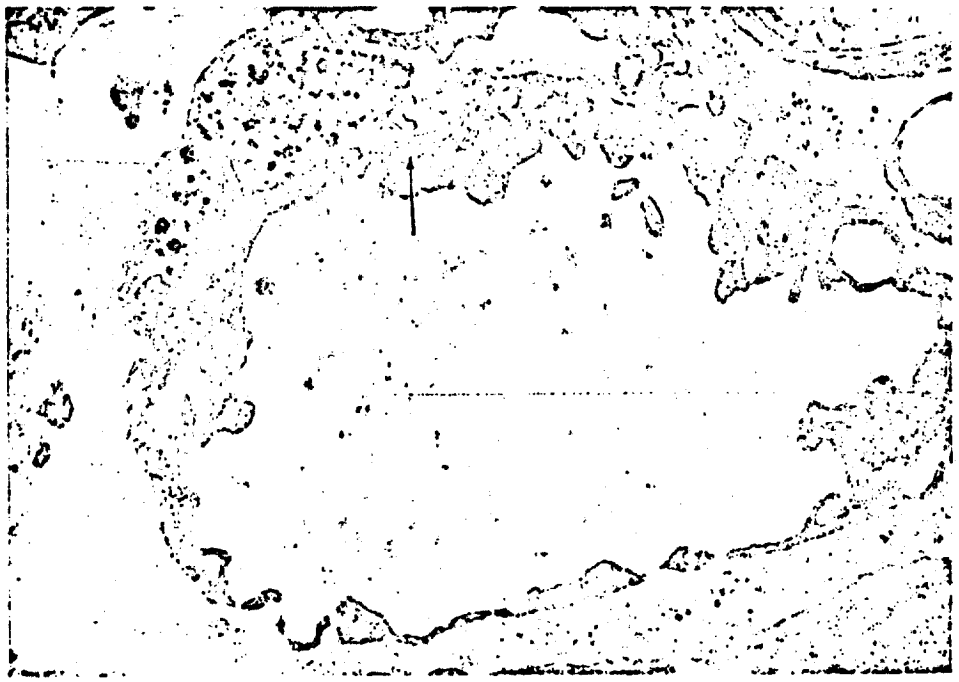

FIG. 9.—EM. Capillary in mucosa of small bowel of rat given carbon immediately after injection of mustard oil and killed 45 min. later. A large intramural carbon deposit lies beneath a closed intercellular junction (↗). Fenestrae are present in opposite side of endothelium, which appears normal. ×21,400.

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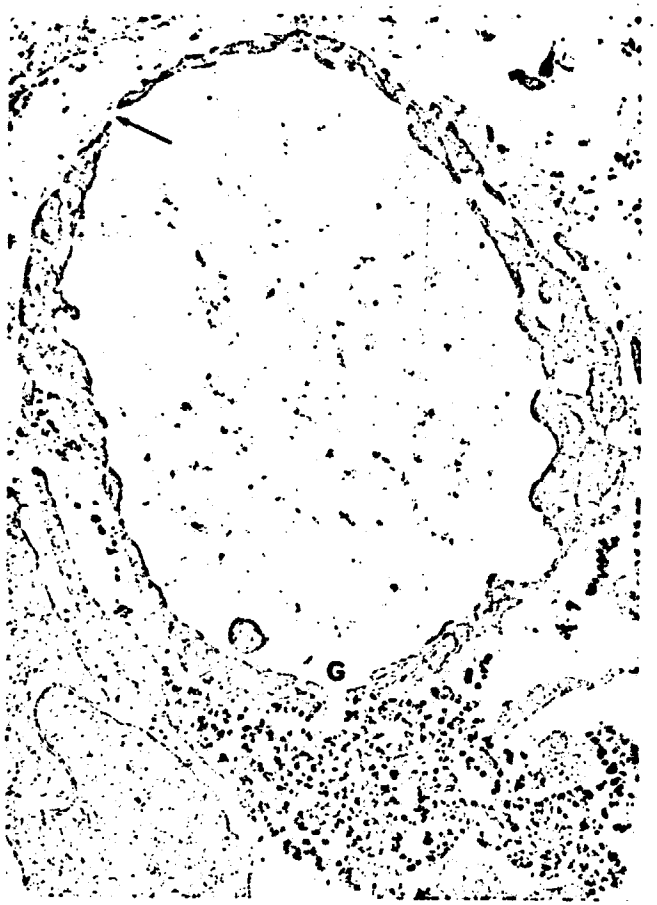

FIG. 10.—EM. Capillary from small-bowel mucosa of rat given injection of carbon immediately after application of mustard oil and killed 45 min. later. A gap (G) is present in endothelium covering an intramural carbon deposit. A single fenestra (↗) is present in opposite side of vessel. Endothelium appears normal. ×16,800.

FIG. 11.—EM. Small vessel from small-bowel mucosa of rat given carbon immediately after mustard oil injection and killed 40 min. later. Two large gaps (between ↗↗) are present in endothelium which is abnormally dense and has lost many organelles. Carbon, ferritin, fibrin and platelets are visible adjacent to the gaps. ×13,150.

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FENESTRATED INTESTINAL VESSELS AND MUSTARD OIL

FIG. 12.—EM. Capillary from small-bowel mucosa of rat given carbon immediately after mustard oil injection and killed 45 min. later. Carbon and ferritin particles lie deep to a gap in endothelium of normal appearance. A closed junction lies adjacent to the gap. Fenestrae were visible in opposite side of this vessel. ×27,600.

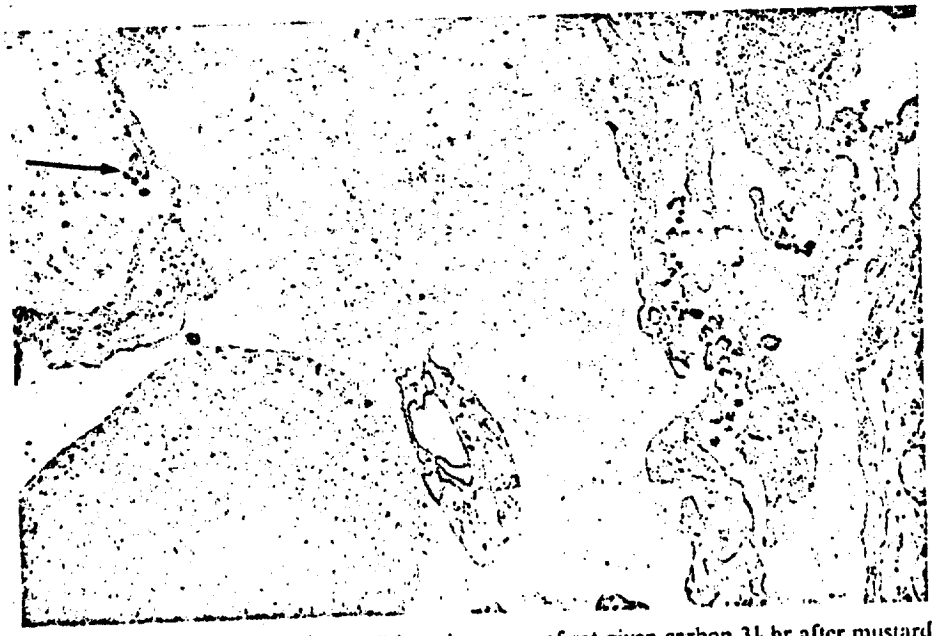

FIG. 13.—EM. Small venule from small-bowel mucosa of rat given carbon 3½ hr after mustard oil injection and killed 50 min. later. A gap overlies a large intramural carbon deposit. Carbon (↗) has also escaped from the opposite side of venule. Endothelium appears denser than normal. ×30,000.

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FENESTRATED INTESTINAL VESSELS AND MUSTARD OIL

FIG. 14.—EM. Venule from small-bowel mucosa of rat given carbon 3½ hr after mustard oil injection and killed 50 min. later. Carbon, a platelet and part of an erythrocyte have escaped on one side, and carbon alone from the other side. Endothelium appears mildly damaged. A leucocyte (L) is present in extravascular tissues. ×12,600.

FIG. 15.—EM. Low-power view of small venule in small-bowel mucosa of rat given carbon 3½ hr after mustard oil injection and killed 50 min. later. Endothelium is severely damaged and contains many large vacuoles. Leucocytes and carbon (↗) are visible deep to damaged endothelium. ×3450.

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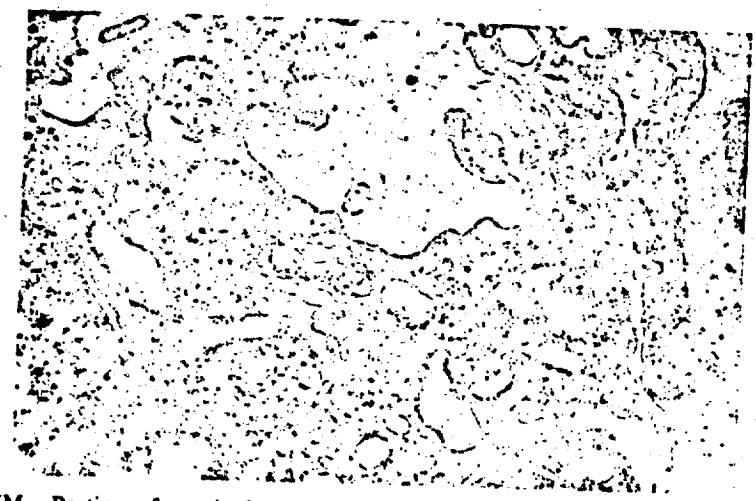

FIG. 16.—EM. Portion of venule from mucosa of small bowel of rat given carbon 2 hr after mustard oil application and killed 48 hr later. Lumen is patent. Endothelium contains dilated endoplasmic reticulum, Golgi elements and free granules of RNA; few pinocytic vesicles are present. ×24,000.

FIG. 17.—EM. Small vessel in small-bowel mucosa of rat given carbon 2 hr after mustard oil and killed 48 hr later. Lumen is patent. Dilated endoplasmic reticulum and prominent rosettes of RNA are visible in endothelial cytoplasm. Pinocytic vesicles are reduced in number. ×27,000.

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distribution and appearance. Leucocytic emigration is visible in some venules and leucocytes are present in extravascular tissues. Many labelled venules and capillaries, some with gaps in their endothelium, are present, and the distribution of carbon and ferritin is similar to that seen 45 min. after injection of mustard oil. No change is apparent in the basement-membrane. The appearances in muscularis and serosa are similar to those seen 45 min. after injury.

Four hours after injury the endothelium of most mucosal and submucosal vessels is abnormal to some degree (figs. 13–15), and in a few vessels almost complete destruction of endothelial cells has occurred. Almost all vessels appear patent with much ferritin and occasional carbon particles visible in the lumen. Occasional vessels are plugged with masses composed of carbon, fibrin and platelets in varying amounts. The proportion of labelled vessels is less than at earlier times, and few endothelial gaps are visible (fig. 13). Active leucocytic emigration is present and some extravascular leucocytes contain ingested carbon particles. Much ferritin is visible in extravascular tissues. Both fenestrae and basement-membrane appear unchanged. Muscularis and serosa appear normal and contain occasional labelled vessels.

The appearances are similar 24 and 48 hr after mustard oil injection. In the mucosa almost all vessels appear patent. Some endothelial cells still appear swollen and pale, but most have an appearance not seen at earlier times (figs. 16 and 17). These cells have an abundant cytoplasm containing few pinocytic vesicles but many mitochondria and Golgi elements. Many particles of RNA are visible, some arranged along cisternae of endoplasmic reticulum and others forming small rosettes. Fenestrae and basement-membrane appear normal. No gaps between endothelial cells are visible and leucocytic emigration has largely ceased. In animals given carbon shortly before they were killed very few labelled vessels are visible: animals given a carbon injection 2 hr after injury show a similar distribution of carbon to that seen 2–3 hr after mustard oil injection. The amount of extravascular ferritin is similar to that found in control animals. No labelling is visible in vessels of the muscularis and serosa, which are of normal appearance.

Effect of injection of permeability factors into loops of small intestine

0·20 ml of histamine acid phosphate at concentrations of 200, 400, 1000 and 2000 μg per ml was injected into loops of small intestine by the method used for injection of mustard oil. Carbon was given intravenously immediately after injection of histamine and the animals were killed 1 hr later. Examination of cleared and histological preparations shows no deposition of carbon in the mucosa except in Peyer's patches where the appearances are identical with those in normal and control animals.

0·20 ml of lymph-node permeability factor (LNPF) (5 mg per ml in saline) was injected in the same way. Again no carbon deposits are visible in mucosa or submucosa.

In all these experiments there is blackening of venules in the muscularis and serosa similar to, but more intense than, that seen in control animals.

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Time-sequence and pattern of vascular labelling after application of mustard oil to skeletal muscle

0·1 ml of mustard oil of the concentration used for bowel injections was injected subcutaneously over each cremaster of six rats. Carbon was given intravenously at several times after mustard oil injection and the animals were killed 1 hr after carbon injection.

In cleared specimens, blackening of both venules and capillaries is visible in a localised area around the mustard oil injection in all specimens. Labelling is equally intense immediately and 2 hr after mustard oil application, is somewhat decreased at 4 hr and has almost disappeared by 6 hr.

In a further experiment a longitudinal incision was made in the anterior abdominal skin, the skin was retracted and two circular disks of filter-paper 1 cm in diameter were applied to the exposed abdominal muscle. One paper had been previously soaked in olive oil and the other in mustard oil solution three times the strength of that used for bowel injections. Carbon was injected intravenously 1 hr later and the animal killed after a further hour.

In cleared specimens of abdominal muscle no labelling is present in the area where the olive oil-soaked paper was applied, but a sharply outlined disk of blackening of venules and capillaries almost precisely 1 cm in diameter is visible where the paper soaked in mustard oil was applied.

Discussion

The increased permeability of mucosal vessels seen after application of mustard oil to the small bowel appears to be due to direct vascular injury. It has all the characteristics of previously described injuries of this type such as mild thermal burns (Cotran and Majno, 1964; Cotran, 1965; Hurley, Ham and Ryan, 1967; Ham and Hurley, 1968), X-ray injury (Hurley, Ham and Ryan, 1969) and mechanical crushing (Hurley and Edwards, 1969). Thus leakage occurs from vessels of all sizes within the injured area and is restricted to the tissues in contact with the irritant. Electron-microscope examination shows damage to endothelial cells maximal several hours after injury and succeeded by characteristic reparative changes 24–48 hr later. The findings after application of mustard oil to skeletal muscle support this conclusion and in particular the localisation of leakage to the area of injury is clearly shown after application of mustard oil-soaked filter-paper to the exposed abdominal muscle.

There appears to be no previous published report of a combined topographical and electron-microscope study of damaged tissue containing fenestrated blood vessels. The present experiments show that the response to injury of fenestrated vessels, at least in small-bowel mucosa, is the same as that now well established for vessels lined by continuous endothelium, i.e., the formation of reversible gaps at the junctions between endothelial cells. In the present experiments a high proportion of the intramural carbon deposits seen lay beneath closed intercellular junctions, and gaps were found in the endothelium covering some intramural carbon deposits. These deposits also contained varying amounts

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of ferritin, indicating that ferritin particles also escape through the gaps and subsequently pass through the basement-membrane and spread widely in extravascular tissues. By contrast, no intramural deposits of carbon or ferritin were seen in relation to fenestrae, and no change was detected in the appearance of fenestrae in either mildly or severely damaged vessels. Florey (1961) previously reported no change in the appearance of fenestrae in the vessels of mouse or rat colon after application of mustard oil. It seems clear from the findings in the present experiments that the large amount of plasma protein that escapes from inflamed fenestrated vessels does so via opened intercellular junctions.

However, the findings provide no evidence of the route of escape of the much smaller amount of protein that leaks normally from fenestrated vessels. It seems that fenestrae in small bowel (Clementi and Palade, 1969*a*) and pancreas (Walters *et al.*, 1970) are permeable to horseradish peroxidase, but the evidence that significant quantities of ferritin also escape in this way is far less convincing (Florey, 1968; Clementi and Palade, 1969*a*). After studying several different organs, Florey (1968) concluded that although fenestrated vessels in various organs have similar morphological characters their physiological performance may not be identical. In preliminary experiments we have found that the rate of escape of colloidal mercuric sulphide, measured both chemically and electron-microscopically, is much greater in small bowel than in submaxillary gland, although the fenestrated vessels appear similar in the two organs. Further studies of fenestrated vessels in different situations, both in a normal state and during acute inflammation, appear to be indicated.

Histamine, even in high dosage, and LNPF appear not to induce increased permeability of either fenestrated or non-fenestrated vessels in small-bowel mucosa. That the injected permeability factors penetrated through the mucosa is shown by the increased blackening of venules in muscularis and serosa seen in these animals. The lesser degree of labelling seen in deeper layers of the bowel wall in all animals, including controls, examined within 2–3 hr of injection of solutions into the bowel lumen appears due to trauma in handling the exposed bowel. The inability of histamine to cause leakage from mucosal vessels is in conflict with the report of Clementi and Palade (1969*b*), who perfused the vessels of isolated loops of bowel with carbon suspensions containing various concentrations of histamine, and claimed that histamine caused removal of the diaphragms of occasional fenestrae and accumulation of carbon against the exposed basement-membrane. They saw no alteration in the intercellular junctions of the perfused vessels. In the present experiments with mustard oil no intramural carbon was seen in relation to fenestrae during careful examination of very many labelled vessels. Confirmation *in vivo* appears necessary before accepting the claimed effect of histamine on fenestral diaphragms.

The heavy carbon labelling of post-capillary venules in Peyer's patches appears to be due to passage of carbon and ferritin down the junctions between the high columnar endothelial cells of these vessels. Neither open junctions nor intracellular marker particles were seen, and many of the particles appear unrelated to emigrating lymphocytes. The leakiness of post-capillary venules

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must lead to escape of a considerable amount of plasma and circulating antibodies as well as lymphocytes from this type of vessel. The immunological significance of this leakage of plasma is not clear. Vessels with high endothelium, similar to post-capillary venules, develop in chronic skin granulomata (Smith, McIntosh and Morris, 1970). Lymphocytes pass out through these vessels into the granulomata, but their permeability to marker particles has not been reported.

SUMMARY

Combined topographical and electron-microscope study shows that application of mustard oil to the mucosa of the small intestine of rats causes increased permeability of both capillaries and venules in the mucosa. The leakage, which starts immediately after injury and lasts for several hours, appears to be due to direct injury to the wall of small blood vessels.

Electron-microscope examination shows that most of the leakage occurs from vessels lined by fenestrated endothelium. In these inflamed fenestrated vessels, escape of both carbon and ferritin particles occurs through reversible gaps which form at the junctions between endothelial cells. Fenestrae appear unaltered and apparently play no part in the increased permeability of the damaged vessels.

No increase in the permeability of mucosal vessels was found after injection of histamine or lymph-node permeability factor into the lumen of the small bowel.

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Coronary Disease Associated With Ingestion of Mustard

JACKSON BLAIR, M.D.

THE following cases of coronary disease associated with the eating of large amounts of table mustard are reported:

1. M. K., male, age 49. At age 45 had coronary thrombosis with infarction. "I ate one sandwich with mustard at least once a day for up to 20 years, and also mustard on eggs, up to three times a day on eggs."

2. J. A. S., male, age 57. In 1963 had acute coronary attack with myocardial infarction and another slight coronary attack in 1964. "I used mustard on sandwiches every day at work and used it at home on bologna or ham sandwiches and also used considerable mayonnaise (which contains mustard)."

3. W. A., male of Polish descent, age 36. Had coronary attack with mild infarction two years ago. "I ate large amounts of mustard on ham and ham sandwiches, on hot dogs; and on hamburgers at least twice weekly, and mayonnaise every day. I used to mix powdered mustard with beer to make it stronger."

4. J. L. B., male of Slovak descent, age 45. Had acute coronary thrombosis and infarction. "I carried my lunch for years, and 90 per cent of the time had mustard on sandwiches, and also used it on hot dogs."

5. H. G., female, age 49. Had acute coronary attack and myocardial infarction. "I ate a sandwich with mustard on it every day for nine years at the shop. I also ate a lot of hot dogs and hamburgers with mustard."

6. W. B., male, age 47. At age 41 had acute coronary thrombosis with infarction. "I used a great deal of mustard as I loved cheese and ham and always put mustard on it. I also used a great deal of mayonnaise."

7. F. J., female, age 59. Died of acute coronary thrombosis and infarction. This history was obtained from her husband: "My wife ate considerable mustard with hot dogs, one or two at picnics which we had very frequently. She earlier used mustard from a jar but lately it comes in tubes and she put it on from tubes. She also used mayonnaise at least twice weekly on salad greens and on potato salad at all picnics."

8. L. K., male, age 48. Had acute coronary thrombosis and infarction. "I ate two or three hot dogs every night with mustard, and I used a lot of pepper on my food and drank a lot of ginger-ale." Six months following his coronary attack, his blood pressure was 185 systolic and 130 diastolic. (This additional use of pepper and ginger-ale are significant and will be commented upon later.)

9. W. L., male, age 54. At age 49 had acute coronary thrombosis with infarction. "I ate lots of mustard, two hamburgers every day, covered with mustard, for two years, and some mustard before that. Since my attack I have not eaten mustard, pepper, or pickled peppers."

The next two patients were nearest neighbors and friends of the author, as well as patients.

10. E. D., male, suffered two coronary attacks with infarction and did not survive his second coronary attack with massive myocardial infarction precipitated by exertion in sub-zero weather. He liked to eat cold meats, always with large amounts of mustard, for luncheon and very frequent evening snacks.

11. F. D., male, suffered an acute coronary attack with infarction. He was very fond of ham and ate it at least once or twice a week over a period of years, always with mustard. He survived his first attack but succumbed to complications, one facet of which was myocardial insufficiency.

12. Another victim of coronary thrombosis with myocardial infarction was a man age 50, whose wife informed me that he ate a large amount of pepper, mustard, ginger, but whose favorite pastime was to sit in front of his television set with a bag of pretzels and a jar of mustard, break off a piece of pretzel, dip the broken end in the mustard, and enjoy TV and pretzels a la mustard.

Submitted February 25, 1965.

The Author

● Dr. Blair, Cleveland, is a member of the medical staffs, Lutheran Hospital, and St. John's Hospital, Cleveland; Lakewood Hospital, Lakewood.

It is the author's opinion that mustard is the dietary agent responsible for the primary injury leading to the coronary atherosclerosis, and probably responsible in some cases to triggering the thrombosis.

What are the steps leading to such a hypothesis and conclusion?

Pepper, Mustard, Ginger, in Hypertension

In 1948 the author[1] reported 50 cases of hypertension in excessive users of the hot condiments pepper, mustard, and ginger and presented the theory that excessive eating of these condiments is a common cause of essential hypertension, that hypertension may be produced experimentally by repeated feeding of these condiments, and that the damage may be irreversible.

In 1957 Roth and Blair[2] produced hypertension in rats by feeding these condiments and showed that the hypertension persisted for months after the condiments were discontinued.

Of pepper, mustard, and ginger, the most irritating appeared to be mustard, which contains not less than .006 per cent of the most poisonous of the volatile oils, allyl isothiocyanate (C_3H_5NCS). The author first suspected the allyl isothiocyanate because in the reports of fatal cases of oil of mustard poison-

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ing the blood and urine had the odor of horse-radish and horse-radish contains the poisonous allyl isothiocyanate.

The author having pinpointed this chemical, Roth and Blair[3] produced hypertension in all experimental rats placed on breeder's diet plus allyl isothiocyanate; and in 1959 the author stated this work appears to open an entirely new approach to the etiology of arteriosclerosis and coronary disease.[3]

Mustard in Atherosclerosis

The initial role of mustard in the production of atherosclerosis was suspected for many reasons.

Sollmann[4] states, "the sharp taste and irritation which black mustard seed develops on contact with water is due to the formation of the volatile oil, allyl isothiocyanate, by the action of the enzyme myrosin on the glucoside sinigrin, and is the most irritating of the volatile oils, and, in pure form should not be touched, tasted or smelled."

The oil of mustard has a strongly injurious effect upon the walls of the capillaries[5] and consequently the mustard burn is very slow of healing.[5]

Mustard is absorbed from the stomach into the blood stream and the previous ingestion of mustard may be detected by the finding of allyl sulfide in the urine.

Thiocyanate was used in the treatment of severe hypertension some years ago but was abandoned for it was associated with severe toxic effect and high incidence of thrombophlebitis. Another example of association of mustard with thrombotic phenomena was the case of attempted abortion with a douche of powdered kitchen mustard in hot water reported by Lovitt[6] where autopsy showed pulmonary embolism, lower nephron lesions, acute ulcerative bronchitis, and microscopically, embolism in the veins of the uterus with early organization and thrombosis and an acute inflammatory exudate in the adventitia of these vessels.

Vesiculation and Ulceration Caused by Mustard

Sollmann[4] produced erythema, then vesiculation and later ulceration of the skin by application of mustard poultices. Since mustard or its active agent allyl isothiocyanate can produce ulceration of the skin, would not a similar ulceration of the intima of an artery (or on a previously produced atheroma), and this ulceration being bathed with blood, produce or favor the production of a thrombus or thrombosis?

Because allyl isothiocyanate is a strongly irritative and corrosive chemical it is probable that one of the earliest sites of its tissue damage, dependent on its concentration, be near the first places reached in the circulatory system, the aorta and coronaries, and where the hemodynamic action of the heart exerts its principal action.

The specific cause of the formation of the atheromatous plaque has been sought for years without success but many observers believe an injury to the intima or blood vessel wall precedes the formation of the atheroma.

Similarity of Atheroma and The Cauliflower Ear

The author believes the formation of the atheroma is not unlike the sequence following the blow to a prize fighter's ear (here a mechanical injury) causing production of a serous swelling or a hemorrhage, followed by fibrin clotting, organization and deposit of lipids, cholesterol and calcium and ending with

EDITOR'S NOTE: If they had been submitted to present-day medical journals, it has been said, Withering's observations on the diuretic properties of foxglove would have been rejected forthwith. Who would accept the uncontrolled observations of a country physician regarding the therapeutic properties of a brew made from the leaves of a common garden flower? Two hundred years later, however, we are still trying to explain the mechanism of action of this remarkable brew.

Who will accept, on the basis of his uncontrolled observations and incomplete documentation, Dr. Blair's contention that the ingestion of mustard plays a role in the pathogenesis of hypertension, atherosclerosis, and intravascular thrombosis? Not I. Yet, the author, a general practitioner from Cleveland, asks only that his observations be given the light of day and that "a few individuals, other than myself, . . . approach the question with an open mind and confirm or refute the laboratory experiments, reviews of clinical cases, and reviews of the epidemiology in the light of my findings."

If it appeared likely that the etiology of the vascular phenomena in question might be fully explained by any of the mechanisms now the subject of much discussion in medical literature, we would have rejected this paper without further ado. However, we find ourselves somewhat in the position of Dr. Richard W. Watts of Rocky River (through whom the manuscript was sent to us), who wrote "I realize the observations are somewhat unusual and personally I can't substantiate them but at least it represents a different viewpoint which may, or may not, be borne out in the experience of others." Accordingly, we have accepted the manuscript essentially as submitted.

Perhaps the reader finds our analogy with Withering and foxglove farfetched or inappropriate. Would he like to try Jenner and cowpox in its place?

— P. R. A.

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the cauliflower ear. The formation of the atheroma probably follows a similar pattern (except the injury is chemical) with vesiculation and perhaps a deeper injury to the capillaries or to the vasa vasorum of Winternitz, and then repair by the deposit of lipids, cholesterol and calcium.

Selectivity of Coronary Disease And Hypertension

Coronary disease is so widespread in this country that anyone would appear to have an opportunity to develop it whatever its cause might be.

The author believes that nowhere save in the allyl isothiocyanate found abundantly in mustard, is there a substance so likely and capable of being the etiological cause of the original injury, and which is so available on a selective basis, for in 1960 over 53,000,000 pounds of mustard were either imported[7] or grown[8] in the United States.

Inheritance by Gens or Inheritance Of Customs

Heredity has been suspect, but heredity is of two parts: inheritance of body from parents and ancestors, and inheritance of culture, customs, and diet. In the case of both hypertension and coronary disease the author believes the gens of heredity have little part, but the inheritance of a diet pattern, or habits of eating, has by far the major influence.

Fallacy of Accepted Conclusions In Epidemiology

Important supports for the cholesterol theory are the reports of reduced coronary disease in Norway and Germany in World War II and the conclusion drawn that the reduction of fats in the diet was responsible. The author believes this conclusion is not true, but rather the true conclusion is that mustard was less available at that time, for shipping problems were too great, and more importantly, the mustard fields of Sweden and Denmark were used to produce food crops and not mustard and, consequently, the lesser use of mustard was responsible for the reduction of coronary disease.

Another case in point is the low incidence of coronary disease in the Roseta Colony of Italians and the fairly high incidence of hypertension. This was to be expected. The Italians in general eat quantities of pepper and therefore have a high level of hypertension, but they are so devoted to the pepper they eat practically no mustard and therefore have very little coronary disease. At least I believe that is the answer to the findings in the Rosetans.

Limits of space preclude listing all of the epidemiological findings, and the clinical, and experimental facts, known to pertain to essential hypertension and coronary disease, but suffice it to say, the whole of this vast evidence appears to fit into the theories, hypotheses and experiments outlined in this paper like a gigantic picture puzzle nearing completion. (Even the humoral mechanisms of hypertension including angiotensin and pressure substances, salt retention, cardiac enlargement, etc., fit into the final picture, but are, the author believes secondary to and follow the initial injury produced by the hot condiments.)

Prevention of Coronary Disease And Hypertension

In conclusion an effort has been made to review previous work with hot condiments; to report cases of coronary thrombosis and myocardial infarction associated with the eating of mustard; to point out the poisonous nature of the allyl isothiocyanate of mustard; and to enumerate its harmful effects on the skin, its absorption from the stomach into the blood stream, its injurious effects on the capillaries, and its burn as being slow in healing.

The author is of the opinion allyl isothiocyanate is the underlying cause of the production of the majority of cases of atheromatous sclerosis, that it has thrombotic properties and may even play a part in triggering some cases of the actual coronary thrombosis, and that the restriction of the hot condiments offers a means of prevention of the majority of the cases of so-called essential hypertension, and coronary disease, and merits the consideration of the clinician, epidemiologist, and laboratory research investigator.

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Research Section

Food Flavourings and Compounds of Related Structure
I. Acute Oral Toxicity

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Abstract—Oral dosages of 107 synthetic and natural flavourings and structurally-related compounds were administered by intubation to the mouse, rat or guinea-pig. Animals were observed usually for 2 weeks during which time the development of toxic signs was followed and time of death recorded. The acute oral LD_{50} of each compound was determined.

INTRODUCTION

Substances used as food flavourings have received little attention from the toxicological viewpoint. Because of their extensive use as food additives, the Food and Drug Administration has been investigating their toxicity.

The initial step in our toxicity studies was the determination of the acute oral effects. This paper presents data on acute toxicity for a large number of flavouring matters. Similar data are reported for additional compounds, not necessarily flavourings, but included as a means of correlating structure with toxicity. These relationships have been discussed by Taylor, Jenner & Jones (1964) and Hagan, Jenner, Jones & Fitzhugh—*Toxicology;* Long, Brouwer & Webb—*Pathology* (1964).

Flavour additives include compounds with a wide variety of chemical structures, and mixtures of variable composition derived from plants and other natural sources. Some of the substances are synthetic, others are isolates or extracts of natural products. Since the purpose of these studies was to evaluate the toxicity of these materials in relation to their use as food additives, a commercially available material was used. No attempt was made to secure chemically pure compounds.

METHODS

Groups of 10 young adult Osborne–Mendel rats evenly divided by sex were fasted for approximately 18 hr prior to treatment. Groups of guinea-pigs consisting of both males and females were fasted for the same period. Mice were treated on full stomachs. Animals had access to water at all times, and the food was replaced in cages as soon as animals received their respective doses. All doses were given by intubation.

All animals were maintained under close observation for recording toxic signs and time of death. Such observation was continued until animals appeared normal and showed weight gain. The usual observation period was 2 weeks; in a few cases, where no acute toxic signs were seen, the animals were observed for only one week. LD_{50}'s were computed by the method of Litchfield & Wilcoxon (1949).

RESULTS

The LD_{50}'s, slope function, and their confidence limits, together with toxic signs and times of death, are recorded for each compound in Table 1. The species tested, solvent, and solution concentration (w/v) are also listed. Where no solvent is indicated the substance was a liquid and it was administered undiluted.

Table. 1 *Acute oral toxicity of food flavourings and compounds of related structure*

Table. 1 *Acute oral toxicity of food flavourings and compounds of related structure*

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
Acetophenone (methyl-1-phenyl ketone)		Rat	3200 (2460–4160)	1·9 (1·3–2·8)	Coma within 5 min persisting in some rats for 24 hr D.T. 1 hr–4 days
Aldehyde C-10 decyl (decanal)		Rat	>33,320*	—	Excitation, diarrhoea, wet fur on stomach and posterior in both species
		Mouse	>41,750	—	
Aldehyde C-14 (γ-undecalactone)		Rat	18,500 (16,930–20,260)	1·2 (1·0–1·3)	Depression within 10 min. Wet fur D.T. 4 hr–5 days
Aldehyde C-18 prunolide (lactone of 4-hydroxy-nonanoic acid)		Rat	9780 (7480–12,810)	1·6 (1·4–1·8)	Depression, coma D.T. 4–18 hr
do.		Guinea-pig	3440 (2890–4100)	1·5 (1·2–1·8)	Depression, salivation D.T. 4 hr–6 days
Allyl acetate†	A‡—10%	Rat	142 (116–175)	1·6 (1·3–2·0)	Depression soon after treatment. Rough fur, scrawny appearance for several days D.T. 4 hr–6 days
do.	A—2 & 5%	Mouse	170 (152–190)	1·2 (1·1–1·3)	Depression D.T. 4–18 hr
Allylacetic acid† (pentenoic acid)	A—15%	Rat	470 (385–573)	1·5 (1·3–1·8)	Depression within 30 min. High dose caused convulsions. Most rats that recover appear normal the day following treatment D.T. 30 min–18 hr
do.	A—15%	Mouse	610 (460–808)	2·2 (1·5–3·1)	Depression D.T. 1–18 hr
Allyl alcohol†	B—2%	Rat	70 (63–79)	1·6 (1·2–2·0)	Depression, colourless secretion from eyes, diarrhoea, scrawny appearance for several days D.T. 4 hr–4 days

*Highest dose administered

†Toxicity was studied because of structural relation to a flavouring agent.

‡A=corn oil; B=water.

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
p-Allylanisole (1-methoxy-4-allylbenzene)		Rat	1820 (1670–1980)	1·2 (1·1–1·3)	Marked depression, some rats in coma for 24 hr, rough fur, wet posterior, porphyrin-like deposit around eyes D.T. 4 hr–8 days
do.	A—10, 20%	Mouse	1250 (812–1920)	1·6 (0·9–2·8)	Depression soon after treatment, coma on higher doses D.T. 1 hr–4 days
Allylbenzene†		Rat	5540 (4620–6650)	1·5 (1·2–2·0)	Depression, some rats comatose for 2–3 days, wet posterior, scrawny appearance for several days D.T. 4 hr–5 days
Allyl butyrate (allyl butanoate)		Rat	250 (216–290)	1·5 (1·2–1·8)	Depression, wet posterior, scrawny appearance for several days D.T. 4 hr–5 days
Allyl caproate (allyl hexanoate)		Rat	218 (186–255)	1·3 (1·1–1·5)	Depression. Scrawny appearance D.T. 4–18 hr
do.		Guinea-pig	280 (246–319)	1·3 (1·1–1·5)	Depression, salivation D.T. 4 hr–3 days
Allyl cinnamate (allyl 3-phenylacrylate)		Rat	1520 (1290–790)	1·4 (1·1–1·8)	Scrawny appearance D.T. 4 hr–8 days
Allyl cyclohexane propionate (allyl 3-cyclohexyl-propionate)		Rat	585 (480–714)	1·5 (1·0–2·2)	Depression, rough fur D.T. 4–hr 6 days
do.		Guinea-pig	380 (172–834)	1·3 (0·5–3·8)	Depression, salivation, haemorrhage in small intestine D.T. 1 hr–6 days
Allyl formate†	A—5%	Rat	124 (107–144)	1·3 (0·5–3·6)	Depression, scrawny appearance for several days D.T. 4 hr–5 days
do.	A—2%	Mouse	136 (122–151)	1·2 (1·1–1·3)	Depression D.T. 4–18 hr
Allyl heptylate (allyl heptanoate)	A—50%	Rat	500 (392–638)	1·7 (1·2–2·2)	Ataxia D.T. 2–18 hr
do.		Guinea-pig	444 (363–541)	1·6 (1·2–2·3)	Depression D.T. 2–18 hr

Table 1 (*Continued*)

Allyl heptylate (allyl heptanoate)	A—50%	Rat	[illegible] (392–638)	1·7 (1·2–2·2)	Ataxia D.T. 2–18 hr
do.		Guinea-pig	444 (3–541)	1·6 (1·2–2·3)	Depression D.T. 2–18 hr

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
do.	A—25%	Mouse	630 (514–772)	1·5 (1·2–1·8)	Depression D.T. 2–18 hr
Allyl isothiocyanate	A—10%	Rat	339 (318–361)	1·6 (1·2–2·1)	Scrawny appearance, porphyrin-like deposit around eyes and nose, rough fur D.T. 4 hr–15 days
Amyl acetate (pentyl acetate)		Rat	16,550 (14,370–19,030)	1·2 (1·1–1·4)	Depression, coma, rough fur D.T. 4 hr–2 days
Amyl alcohol (pentanol)		Rat	3030 (1440–6360)	1·2 (1·0–1·4)	Depression D.T. few min–18 hr
Amyl butyrate (pentyl butyrate)		Rat	12,210 (10,260–14,560)	1·6 (1·3–1·8)	Depression, rough fur, wet posterior D.T. few min–2 hr
do.		Guinea-pig	11,950 (8530–16,730)	3·0 (1·2–7·2)	Depression, ataxia D.T. 2 hr–6 days
Amyl cinnamic aldehyde (α-*n*-pentyl-β-phenyl-acrolein)		Rat	3730 (3190–4370)	1·4 (1·2–1·6)	Depression, porphyrin-like deposit around eyes and nose D.T. 4 hr–5 days
Amyl valerianate (pentyl pentanoate)		Rat	>35,420*	—	No effect in 2 weeks observation
do.		Guinea-pig	>17,260*	—	Wet fur D.T. 2–6 days
Amyris oil		Rat	5580 (4540–6860)	1·5 (1·4–1·6)	Ataxia, coma within 1 hr, porphyrin-like deposit around eyes and nose. Wet posterior D.T. 1–3 days
Anethole (1-methoxy-4-propenyl-benzene)		Rat	2090 (1420–3070)	1·8 (1·3–2·4)	Low doses—depression, high doses—coma D.T. 4 hr–4 days
do.		Guinea-pig	2160 (1920–2450)	1·3 (1·2–1·5)	Depression D.T. 1–7 days
do.		Mouse	3050 (2330–4000)	1·6 (1·2–2·1)	Depression, coma within 15 min D.T. 2–4 hr
Anisaldehyde (*p*-methoxybenzaldehyde)		Rat	1510 (1360–1700)	1·2 (1·1–1·3)	Depression D.T. 4–18 hr
do.		Guinea-pig	1260 (937–1700)	1·6 (1·2–2·0)	Depression within 2 hr D.T. 1–3 days

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
Anisole (methoxybenzene)		Rat	3700 (3240–4220)	1·2 (1·1–1·4)	Depression, porphyrin-like deposit around eyes, salivation, bloody urine, rough fur D.T. 4 hr–8 days
β-naphthyl *iso*butyl ether		Rat	5930 (4750–7420)	1·9 (1·4–2·5)	Wet posterior, coma within 1 hr, rough fur and black, soft stools D.T. 1–6 days
Benzaldehyde		Rat	1300 (1110–1540)	1·4 (1·2–1·6)	Depression, coma on higher doses D.T. 4–18 hr
do.		Guinea-pig	1000 (800–1250)	1·4 (1·2–1·8)	Diuresis, tremors, intestinal irritation and haemorrhage D.T. 1 hr–4 days
Benzene†		Rat	4080 (3260–5100)	1·7 (1·4–2·1)	Tremors, loss of equilibrium and comatose on high doses, scrawny appearance for several days D.T. 1 hr–4 days
Benzyl acetate		Rat	2490 (2040–3040)	1·6 (1·1–2·4)	Depression D.T. 4 hr–3 days
Benzyl alcohol		Rat	1230 (1130–1330)	1·2 (1·1–1·3)	Depression, coma within 10–15 min. Excitable for 3–4 days D.T. 1 hr–4 days
do.	A—25%	Mouse	1580 (1410–1770)	1·2 (1·1–1·3)	Depression D.T. 2–18 hr
Benzyl *n*-butyrate (benzyl butanoate)		Rat	2330 (1940–2800)	1·3 (1·1–1·6)	Depression, scrawny appearance, tremors with higher doses D.T. 4 hr–4 days
Benzyl cinnamate (cinnamein, benzyl β-phenylacrylate)		Rat	5530 (3100–7740)	2·2 (0·9–3·6)	Depression, coma persisting in some rats for 24 hr D.T. 4 hr–5 days
do.		Guinea-pig	3760 (2340–6055)	4·2 (1·3–13·8)	Depression, gastro-intestinal tract irritation, rectal bleeding D.T. 4 hr–6 days
n-Butyl alcohol (butanol)		Rat	2510 (2220–2840)	1·4 (1·2–1·7)	Depression, coma D.T. 4–18 hr

Table 1 (*Continued*)

Concentration [illegible]	LD_{50} with [illegible]	Slope [illegible]

(butanol)		Rat	2510 (20–2840)	1·4 (1·2–1·7)	Depression, coma D.T. 4–18 hr

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
Cajeput oil		Rat	3870 (3360–4450)	1·2 (1·1–1·3)	Scrawny appearance, wet posterior. Gross pathology shows pale, nutmeg livers. Depression, persisting in some animals for as long as 3 days D.T. 4 hr–9 days
Calamus oil		Rat	777 (612–987)	1·7 (1·3–2·3)	Severe tremors from 30 min–2 hr after treatment. Scrawny appearance, weight loss for several days D.T. 4 hr–8 days
n-Caprylic acid (octanoic acid)		Rat	10,080 (8190–12,370)	1·6 (1·3–2·0)	Depression, diarrhoea D.T. 4 hr–9 days
Carvacrol (2-methyl-5-*iso*propylphenol)		Rat	810 (710–920)	1·2 (1·1–1·3)	Depression within 10 min, coma within 1 hr D.T. 1 hr–3 days
Carvone (*p*-mentha-6, 8-dien-2-one)		Rat	1640 (1260–2130)	1·8 (1·3–2·5)	Depression, ataxia (loss of use of hind legs) D.T. 4 hr–5 days
do.		Guinea-pig	766 (603–845)	1·3 (1·1–1·6)	Extreme depression D.T. 4–18 hr
Cedryl acetate		Rat	44,750 (33,650–59,520)	1·6 (1·3–2·0)	Depression, scrawny appearance, rough, wet fur D.T. 4 hr–11 days
Cinnamic aldehyde (cinnamaldehyde)		Rat	2220 (1910–2600)	1·4 (1·2–1·6)	Depression, diarrhoea, scrawny appearance D.T. 2–3 hr
do.		Guinea-pig	1160 (955–1420)	1·5 (1·2–1·9)	Coma with higher doses D.T. 2 hr–4 days
Citraconic acid (*cis*-methylbutenedioic acid)	B—25%	Rat	1320 (1070–1640)	1·6 (1·1–2·4)	Depression, scrawny appearance, tremors, stomach haemorrhage D.T. 4 hr–3 days
do.	B—25%	Mouse	2260 (1930–2640)	1·4 (1·2–1·6)	Depression, laboured respiration, haemorrhage in gastro-intestinal tract D.T. few min–3 days

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Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
do.	B—25%	Guinea-pig	1350 (1040–1755)	1·9 (1·1–3·1)	Scrawny appearance, gastro-intestinal tract irritation D.T. few min–3 days
Citral (3,7-dimethyl-2,7-octadienal)		Rat	4960 (3940–6240)	1·5 (1·2–2·0)	Depression D.T. 4 hr–4 days
Coumarin§ (2 H-1-benzopyran-2-one)	A—5%	Rat	680 (505–920)	1·8 (1·0–3·5)	Depression soon after treatment, mottled livers D.T. 4 hr–8 days
do.	C‡—10%	Guinea-pig	202 (179–228)	1·2 (1·1–1·4)	Depression, ataxia, severe gastro-intestinal tract irritation D.T. 1–6 days
Cuminaldehyde (*p-iso*propylbenzaldehyde)		Rat	1390 (1140–1700)	1·5 (1·3–1·8)	Depression within 1 hr after treatment, scrawny appearance, porphyrin-like deposit around eyes and nose. Gross pathology showed discoloured liver, irritated gastro-intestinal tract, stomach haemorrhage, yellowish material attached to the intestines D.T. 4 hr–10 days (most deaths within 2 days)
Cyclamen aldehyde (*p-iso*propyl-α-methylhydrocinnamaldehyde)		Rat	3810 (3080–4730)	1·7 (1·3–2·2)	Ataxia soon after treatment, coma within 1 hr. Wet fur, porphyrin-like deposit around eyes and nose D.T. 1–6 days
p-Cymene (4-*iso*propyl-1-methyl benzene)		Rat	4750 (3720–6060)	1·7 (1·5–2·0)	Depression soon after dosing, coma, bloody lacrimation, diarrhoea. Irritable, scrawny appearance for as long as 2 weeks D.T. 4 hr–12 days
Diacetyl (2,3-butanedione)		Rat	1580 (1310–1920)	1·5 (1·2–1·9)	Depression followed by convulsions within 10–15 min after treatment D.T. few min–2 hr

‡C=Propylene glycol

§Not acceptable for food use because of toxic effects observed on chronic feeding.

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§Not acceptable for food use because of toxic effects observed on chronic feeding.

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
do.		Guinea-pig	990 (728–1350)	2·4 (1·2–4·6)	Ataxia, gasping, coma D.T. few min–4 days
Diallylacetic acid†	B—2%	Rat	570 (467–695)	1·6 (1·3–1·9)	Depression soon after treatment, weight loss, scrawny appearance D.T. 3–5 days
Dihydroanethole (1-methoxy-4-propylbenzene)		Rat	4400 (3380–5720)	1·9 (0·6–5·6)	Depression, wet posterior D.T. 4 hr–3 days
do.		Mouse	7300 (5930–9000)	1·6 (1·4–1·8)	Porphyrin-like deposit around eyes and nose, rough fur, scrawny appearance D.T. 1–4 days
Dihydrocoumarin (1,2-benzohydropyrone)		Rat	1460 (1180–1820)	1·6 (1·0–2·6)	Depression D.T. 2 hr–2 days
do.		Guinea-pig	1760 (1460–2170)	1·6 (1·2–2·1)	Depression D.T. 4 hr–4 days
Dihydrosafrole§ (1,2-methylenedioxy-4-propylbenzene)		Rat	2260 (1840–2780)	1·7 (1·4–2·0)	Depression soon after treatment, scrawny appearance, lacrimation D.T. 2–5 days
do.		Mouse	4300 (3420–5420)	1·8 (1·2–2·7)	Severe ataxia, coma D.T. 4 hr–5 days
Dimethylbenzyl carbinol (1,1-dimethyl-2-phenyl-ethanol)		Rat	1280 (934–1770)	2·3 (1·0–5·1)	Depression, coma D.T. 1–24 hr
do.		Guinea-pig	988 (705–1380)	1·7 (1·4–2·0)	Diuresis, coma. Severe gastro-intestinal tract irritation D.T. 1 hr–4 days
Dipropylacetic acid† (2-propylvaleric acid)	B—2%	Rat	670 (598–750)	1·2 (1·0–1·4)	Depression, scrawny appearance, diarrhoea in 1 hr D.T. 2 hr–2 days
Dolcourin‖		Rat	924 (825–1030)	1·2 (1·1–1·3)	Depression, abdominal cavity filled with bloody fluid D.T. 2 hr–5 days
Ethyl butyrate (ethyl butanoate)		Rat	13,050 (12,210–13,940)	1·2 (1·0–1·3)	Depression within a few min, coma on higher doses D.T. 4–18 hr

‖Dolcourin is a trademark name for a substance supplied by Dodge & Olcott, Inc. as a coumarin substitute.

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
Ethyl caprylate (ethyl pentanoate)		Rat	25,960 (22,190–30,370)	1·4 (1·2–1·6)	Depression, coma, wet posterior D.T. 4 hr–4 days
Ethyl formate		Rat	1850 (1520–2240)	1·6 (1·3–1·9)	Depression within 5–10 min. Laboured respiration D.T. 15 min–2 hr
do.		Guinea-pig	1110 (887–1390)	2·0 (1·3–3·1)	Depression. Irritated gastro-intestinal tract D.T. 10 min–2 hr
Ethyl methylphenyl glycidate (ethyl ester of 2,3-epoxy-3-methyl-3-phenylproprionate)		Rat	5470 (4670–6410)	1·3 (1·1–1·5)	Depression, rough fur, porphyrin-like deposit around eyes and nose D.T. 4 hr–8 days
do.		Guinea-pig	4050 (3540–4620)	1·5 (1·2–1·8)	Depression in varying degrees, rectal bleeding on high doses D.T. 4 hr–7 days
Ethyl oenanthate (ethyl heptanoate)		Rat	>34,640*		Depression, coma, rough and wet fur
Ethyl oxyhydrate¶		Rat	14,700 (13,300–16,240)	1·1 (0·9–1·4)	Coma within 5 min D.T. 4 hr–5 days
Ethyl pelargonate (ethyl nonanoate)		Rat	>43,000*	—	Depression, irritated gastro-intestinal tract. Appeared normal 24 hr after treatment
do.		Guinea-pig	24,190 (19,350–30,240)	1·5 (1·2–2·0)	Depression, irritated gastro-intestinal tract D.T. 4 hr–6 days
Ethyl sebacate (ethyl decandioate)		Rat	14,470 (12,805–16,350)	1·4 (1·3–1·5)	Wet fur, depression D.T. 4 hr–4 days
do.		Guinea-pig	7280 (5970–8900)	1·3 (1·1–1·4)	Depression, diuresis D.T. 8 hr–6 days
Ethyl vanillin (3-ethoxy-4-hydroxy-benzaldehyde)	C—20%	Rat	>2000**	—	Depression, coma on high doses D.T. 3–18 hr

¶Ethyl oxyhydrate is a mixture whose composition varies with the method of manufacture. The material used in this study was obtained from Florasynth Labs., Inc., N.Y.

**Toxicity of the propylene glycol solvent prohibited administration of higher doses.

[illegible] hydrate is a mixture whose composition varies with the method of manufacture. The material used in this study was obtained from Florasynth Labs., Inc., N.Y.

**Toxicity of the propylene glycol solvent prohibited administration of [illegible] doses.

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
Eucalyptol (1,8-epoxy-*p*-menthane)		Rat	2480 (2100–2930)	1·4 (1·2–1·6)	Depression, coma on high doses, scrawny appearance for 3–4 days. Recovery within 7 days D.T. 2 hr–4 days
Eugenol (1-hydroxy-2-methoxy-4-allylbenzene)		Rat	2680 (2420–2970)	1·2 (1·1–1·4)	Coma soon after treatment D.T. approximately 1 hr
do.		Mouse	3000 (2400–3750)	1·8 (1·3–2·3)	Severe depression immediately after treatment D.T. few min–2 days
do.		Guinea-pig	2130 (1860–2430)	1·3 (1·2–1·5)	Depression D.T. 4 hr–3 days
Eugenol acetate (1-acetoxy-2-methoxy-4-allylbenzene)		Rat	1670 (1265–2200)	1·9 (1·4–2·5)	Rough fur, depression D.T. 4 hr–2 days
Eugenol methyl ether (1,2-dimethoxy-4-allylbenzene)		Rat	1560 (1170–2070)	2·6 (1·6–4·3)	Coma within 1 hr after treatment D.T. less than 8 hr
Fenchone (*d*,1,3,3-trimethyl-2-norcamphanone)		Rat	6160 (4400–8630)	2·7 (1·1–6·5)	Depression, scrawny appearance, porphyrin-like deposit around eyes and nose for a week after treatment D.T. 4 hr–9 days
2-Furaldehyde	A—5%	Rat	127 (110–147)	1·3 (1·2–1·5)	Depression soon after treatment. Scrawny appearance, porphyrin-like deposit around eyes and nose. Gross pathology showed lung haemorrhage on the higher doses D.T. 4 hr–4 days
Geraniol extra (3,7-dimethyl-2,6-octadienol) (3,7-dimethyl-1,6-octadienol)		Rat	3600 (2840–4570)	1·7 (1·3–2·2)	Depression, coma, wet fur D.T. 4–18 hr
Geranyl acetate		Rat	6330 (5450–7340)	1·3 (1·2–1·4)	Depression, coma D.T. 4 hr–3 days
Geranyl butyrate		Rat	10,660 (8020–14,180)	1·6 (0·7–3·7)	Marked depression and coma on higher doses D.T. few min–4 days

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
Guaiac gum	A—20%	Rat	>5000*	—	Rough fur, scrawny appearance D.T. less than 18 hr
Ho leaf oil		Rat	3270 (2780–3830)	1·6 (1·3–2·0)	Coma on higher doses within 30 min. Scrawny appearance, rough fur, porphyrin-like deposit around eyes and nose D.T. 1–4 days
Hydratropic aldehyde (2-phenylpropanal)		Rat	2800 (2390–3280)	1·3 (1·2–1·4)	Coma within 20 min after treatment. Rough fur D.T. 1–5 days
Ionone [60% α-ionone, 4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one and 40% β-ionone, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one]		Rat	4590 (3880–5400)	1·4 (1·3–1·5)	Depression, tremors D.T. 4 hr–4 days
Isoamyl formate		Rat	9840 (7720–12,550)	1·7 (1·3–2·3)	Depression soon after treatment, scrawny appearance D.T. 4 hr–4 days
Isoeugenol (1-hydroxy-2-methoxy-4-propenylbenzene)		Rat	1560 (1290–1880)	1·4 (1·3–1·5)	Coma soon after treatment, scrawny appearance D.T. 1 hr–7 days
do.		Guinea-pig	1410 (1130–1780)	2·0 (1·5–2·7)	Depression, coma D.T. 3–6 days
Isosafrole§ (1,2-methylenedioxy-4-propenylbenzene)		Rat	1340 (1140–1590)	1·4 (1·2–1·7)	Depression, coma, rough fur, scrawny appearance D.T. 4 hr–8 days
do.	A—25%	Mouse	2470 (2010–3040)	1·7 (1·3–2·2)	Depression soon after treatment. Severe ataxia within 24 hr. Recovery from ataxia but scrawny in appearance within 48 hr D.T. 1 hr–4 days

					... ataxia but scrawny appearance within 48 hr D.T. 1 hr–4 days

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
Juniper tar		Rat	8014 (6550–9770)	1·5 (1·2–1·9)	Depression soon after treatment, scrawny appearance for several days. Irritated gastro-intestinal tract D.T. 4 hr–4 days
Linalool (3,7-dimethyl-1,6-octadien-3-ol or 3,7-dimethyl-1,7-octadien-3-ol)		Rat	2790 (2440–3180)	1·3 (1·2–1·4)	Ataxia soon after treatment D.T. 4–18 hr
Linalyl acetate		Rat	14,550 (12,300–17,170)	1·8 (1·3–2·6)	Depression soon after treatment, coma, wet posterior D.T. 4 hr–4 days
do.		Mouse	13,360 (11,920–15,000)	1·2 (1·1–1·3)	Depression within 10–15 min after treatment D.T. 1–3 days
Linalyl cinnamate		Rat	9960 (8230–12,050)	1·4 (1·2–1·7)	Depression. Scrawny appearance, porphyrin-like deposit around eyes and nose, wet fur on posterior 24 hr after treatment D.T. 4 hr–5 days (most deaths in 48 hr)
Linalyl *iso*butyrate		Rat	>36,300*	—	Depression, wet fur, diarrhoea. Appeared normal after 1 week
do.		Mouse	15,100 (12,330–18,500)	1·6 (1·1–2·2)	Depression soon after treatment. Excitable after 1 hr. Rough fur D.T. 4 hr–3 days
Mace oil		Rat	3640 (3170–4190)	1·4 (1·0–2·0)	Depression. Scrawny appearance for several days after treatment D.T. 4 18 hr
Melilotic anhydride (anhydride of *o*-hydroxy-cinnamic acid)		Rat	1510 (1310–1750)	1·3 (1·1–1·6)	Depre sion D.T. 1 hr–2 days
Menthol (*p*-menthan-3-ol)	A—50%	Rat	3180 (2790–3620)	1·3 (1·1–1·5)	Ataxia, scrawny appearance D.T. 4 hr–3 days

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
Methyl anthranilate (*o*-aminobenzoic acid, methyl ester)		Rat	2910 (2500–3400)	1·4 (0·9–2·0)	Depression, coma D.T. 1–2 days
do.		Mouse	3900 (3260–4680)	1·5 (1·3–1·7)	Depression D.T. 4–18 hr
do.		Guinea-pig	2780 (2210–3500)	1·8 (1·4–2·3)	Depression, gasping, rapid respiration, irritated gastro-intestinal tract D.T. 4 hr–4 days
Methyl benzoate		Rat	1350 (1290–1410)	1·1 (0·7–1·5)	Depression, porphyrin-like deposit around nose, rough fur, wet posterior. Survivors excitable D.T. 2–18 hr
do.	A—50%	Mouse	3330 (2920–3800)	1·2 (1·1–1·4)	Excitation, tremors D.T. few min–18 hr
Methylenedioxybenzene†		Rat	580 (487–690)	1·4 (1·2–1·7)	Depression and coma on high doses, rough fur, porphyrin-like deposit around eyes and nose D.T. 1–6 days
do.	A—25%	Mouse	1220 (976–1520)	1·4 (1·2–1·7)	Depression and coma on high doses, rough fur, porphyrin-like deposit around eyes and nose D.T. 1–5 days
Methyl salicylate (*o*-hydroxybenzoic acid, methyl ester)		Rat	887 (715–1100)	1·5 (1·2–1·8)	Depression soon after treatment D.T. 4–18 hr
do.		Guinea-pig	1060 (873–1300)	1·6 (1·3–1·9)	Convulsions. Irritated gastro-intestinal tract D.T. 1 hr–3 days
Musk ambrette (artificial) (6-*tert*-butyl-3-methyl-2, 4-dinitroanisole)	A—25%	Rat	339 (283–408)	1·4 (1·2–1·7)	Increased respiration and hypersensitivity 24 hr after treatment. Scrawny appearance, wet posterior, rough fur D.T. 1–3 days

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
Nutmeg oil		Rat	2620 (2200–3120)	1·3 (1·2–1·5)	Depression soon after receiving treatment, scrawny appearance for several days D.T. 4–18 hr
Phenylethyl alcohol (2-phenylethanol)		Rat	1790 (1580–2020)	1·2 (1·1–1·3)	Coma within 15 min. Gross pathology shows irritation of the lower half of the stomach on the higher doses D.T. 4–18 hr
Phenylethyl phenylacetate		Rat	15,390 (12,830–18,470)	1·5 (1·4–1·7)	Depression soon after treatment, scrawny appearance for several days D.T. 4 hr–5 days
Piperonal (3,4-methylenedioxybenzaldehyde)	A—25%	Rat	2700 (2350–3100)	1·5 (1·1–2·0)	Tremors for several hr followed by depression and ataxia D.T. 2 hr–5 days
n-Propanol		Rat	6500 (5800–7280)	1·2 (1·1–1·3)	Coma soon after treatment, scrawny appearance D.T. 2–18 hr
Propenylbenzene†		Rat	3600 (2650–4900)	2·2 (1·3–3·8)	Scrawny appearance, weight loss, wet posterior for 5–7 days after treatment D.T. 4 hr–9 days
n-Propyl acetate		Rat	9370 (7670–11,430)	1·5 (1·1–1·8)	Depression soon after treatment, rough fur, scrawny appearance D.T. 4–18 hr
do.		Mouse	8300 (7280–9460)	1·2 (1·1–1·4)	Depression soon after treatment D.T. few min–18 hr
Propylbenzene†		Rat	6040 (4830–7550)	1·5 (1·2–2·0)	Depression soon after receiving treatment. Scrawny appearance D.T. 1–3 days
n-Propyl *n*-butyrate		Rat	15,000 (12,600–17,850)	1·4 (0·8–2·5)	Depression soon after treatment followed by coma on higher doses. Rough fur, diarrhoea D.T. 1–3 days
n-Propyl formate		Rat	3980 (3350–4740)	1·5 (1·2–1·7)	Depression soon after treatment D.T. 4–18 hr

Table 1 (*Continued*)

Compound	Concentration (w/v) and solvent used	Species	LD_{50} with 95% confidence limits (mg/kg)	Slope function with 95% confidence limits	Toxic signs and death time (D.T.)
do.		Mouse	3400 (3060–3780)	1·7 (1·3–2·2)	Depression soon after treatment D.T. few min–6 hr
Safrole§ (1,2-methylenedioxy-4-allylbenzene)		Rat	1950 (1760–2160)	1·3 (0·6–3·0)	Depression, ataxia, diarrhoea D.T. 1–5 days
do.	A—50%	Mouse	2350 (2010–2750)	1·4 (1·1–1·6)	Ataxia, depression, scrawny appearance D.T. 4 hr–7 days
Terpinyl acetate (*p*-menth-1-en-8-ol ester of acetic acid)		Rat	5075 (4160–6190)	1·5 (1·3–1·7)	Depression, scrawny appearance, porphyrin-like deposits around eyes and nose D.T. 4 hr–5 days
Thymol (*p*-cymen-3-ol,3-hydroxy-*p*-cymene)	C—20%	Rat	980 (817–1180)	1·6 (1·3–2·0)	Depression, ataxia, coma on high doses D.T. 4 hr–5 days
do.	C—20%	Guinea-pig	880 (740–1050)	1·6 (1·2–2·1)	Irritated gastro-intestinal tract, tremors, coma, respiratory failure D.T. 1 hr–10 days
Vanillin (4-hydroxy-3-methoxybenzaldehyde)	C—20%	Rat	1580 (1390–1810)	1·3 (1·2–1·5)	Coma soon after treatment D.T. 4 hr–4 days
do.	C—20%	Guinea-pig	1400 (1310–1500)	1·1 (1·09–1·2)	Depression within 1 hr D.T. 1–3 days
Veratrole (1,2-dimethoxybenzene)		Rat	1360 (980–1870)	1·6 (1·3–1·9)	Coma within 10 min after treatment. Salivation, porphyrin-like deposit around eyes, diarrhoea, scrawny appearance for 3–4 days D.T. 1–4 days
do.	A—25%	Mouse	2020 (1650–2480)	1·7 (1·4–2·0)	Rapid, laboured respiration; lacrimation; pawing about mouth; hyperactivity followed by coma within 15 min D.T. few min–4 hr

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Condiments et Complexes de Structure Voisine. I. Toxicité Aiguë par Voie Buccale

Résumé—On administra par intubation des doses de complexes faits de 107 condiments synthétiques et naturels, de structure chimique voisine, à des souris, des rats et des cobayes. On observa habituellement les animaux pendant 2 semaines, durant lesquelles on suivit le développement de signes toxiques et on nota la date de la mort. Pour chaque complexe on détermina la dose orale limite au-delà de laquelle commence l'intoxication aiguë.

Lebensmittelgeschmackszusatze und Verbindungen verwandter Strucktuen I. Akute Oraltoxititat

Zusammenfassung—107 synthetischen un natürliche Geschmackszusätze und strukturverwandte Verbingdungen wurden durch Intubation an Mäuse, Ratten und Meerschweinchen verabreicht. Die Tiere wurden gewöhnlich 2 Wochen lang unter Beobachtung gehalten, während welcher Zeit die Entwicklung toxischer Symptome verfolgt und die Zeit des Todeseintritts registriert wurde. Die akute orale mittlere tödliche Dosis jeder Verbindung wurde festgestellt.

(German publication, pp. 505-7)

Studies on the bacteriostatic action of some mustard oils

By Dr. P. Klesse and Dr. P. Luoschek

In the systematic study of higher plants for bacteriostatic substances, Winter and Willeke (1, 2, 3) found, in 1952, in nasturtium (Tropaeolum maius L.) and in upland cress (Lepidium sativum L.), later also in horse-radish (Cochlearia armoracia), volatile, bacteriostatic substances which, after passage through the body, were effective also in the urine in the same manner and intensity. While the plant itself is inactive, after maceration of the cells the active substance is liberated, apparently by an enzyme. The substance can be distilled with steam and extracted with ether. After evaporation of the ether, Winter (5) obtained an oil which for weeks suppressed all bacterial growh in the "gas test". As constituent of the essential oils, of the "huille aetherée" which according to Lefebure' Traité de Chimie (Paris 1660) yields upon distillation with water "une eau spiritueuse", specifically of the two types of cress and of the horse-radish (Sinigrain mustard oils were known, so that comparative studies with mustard oils appeared justified. In recent years, O. E. Schultz, Gmelin (7, 8, 9) and Barthold (10,11) have examined the mustard oil glycosides by paper chromatography and by electrophoresis, have separated them, in part prepared them in pure form, and have there by greatly extended our knowledge in this field. From the comparison of the actic of nasturtium and of upland cress with respect to their content of benzyl mustard oil, Winter was able to rule out benzyl mustard oil as the active component, just as Winter and Hornbostel (4) ruled out allyl mustard oil for horse-radish.

Our studies covered the synthesis and the determination of the activity and to icity of the following mustard oils: Methyl and ethyl mustard oil as saturated aliphatic, allyl mustard oil as unsaturated aliphatic, phenyl mustard oil as aromatic, and benzyl and phenylethyl mustard oil as aliphatic-aromatic mustard oil.

Ch_3-N=C=S methyl mustard oil

C_2H_3-N=C=S ethyl mustard oil

$CH_2=CH-CH_2-N=C=S$ allyl mustard oil

$-N=C=S$ phenyl mustard oil

$-CH_3-N=C=S$ benzyl mustard oil

$-CH_2-CH_2-N=C=S$ phenylethyl mustard oil

The methyl and ethyl mustard oils were synthesized according to A. W. Hofmann (12) by boiling down the heavy-metal salt of N-methyl (or ethyl)-dithiocarbamic acid. The synthesis of the benzyl and phenylethyl mustard oils was effected with good yield according to J. v. Braun (13) and H. Deutsch via the thiuram disulfide (*).

Methodology of the bacteriostatic test

In the "gas test" according to Winter & Willeke: Two covers of Petri dishes of equal size were placed one on the other so that the upper contained the agar layer inoculated with various bacteria (10 cc), while the lower dish was charged with the substance to be tested, either pure or, in order to be able to test still smaller quantities, in alcohol, ether, or oily solution. The quantities of solvent (alcohol, ether) were selected so that they themselves exerted no inhibiting and no additive action. As test bacteria there were used generally Staphylococcus aureus SG 511, E. coli, and Sarcina lutea. The dishes were then sealed hermetically with scotch tape and incubated for 18 h at 37°. The gas space enclosed by the dishes averaged 180 cc. We used meat extract pepton agar. In addition, each substance was tested in the tube test against Staphylococcus aureus (SG 511). For this, 4.5 cc of glucose bouillon and mustard oil to be tested were filled in a test tube of 22 cc, which was sealed after inoculation with a constant germ quantity. After 15 h of incubation at 37°, a reading was taken to determine which tube of the dilution series was not yet clouded and which was clouded as much as the control without addition of mustard oil.

For the evaluation of the concentration in the gas test, it was assumed that the mustard oil is distributed uniformly in the air space and in the agar. A possible concentration of the mustard oil in the agar was checked according to

Winter. An agar plate was steamed with a certain concentration of benzyl mustard oil (24 h), then used as the lower plate in the gas test. Result: No substantia concentration in the agar. For the calculations of the limit concentration in th tube test, the diffusion of the mustard oil in the air space was not taken into account. Assuming uniform distribution, the results of Table 2 would have to be multiplied by 5 for the limit concentration. The volatility of the mustard oil i essential for the result. In the gas test the easily volatile (aliphatic) mustar oils are more active than in the tube test. Their solution in oil produces the effect of an ether solution. In the gas test, phenyl ether mustard oil shows greater activity in ether solution, a greatly reduced activity in oily solution, whereas in the tube test it is much better. Benzyl mustard oil shows only a moderate absorption of sperm oil.

All mustard oils were still bacteriostatic even when highly diluted.

The limit concentrations for complete inhibition are, depending on the mustard oil and bacterial species, between 1:10,000 and 1:600,000. The aromatic mustard oil - phenyl mustard oil - was much less active than the others, which show value of approximately equal order of magnitude. E. coli is less sensitive to phenylethyl mustard oil than Staphylococcus aureus SG 511 and Sarcina lutea.

Table 1. Testing of bacteriostasis in the gas test.

Product	Staph. aureus		E. coli		Sarcina lutea	
	a	b	a	b	a	b
Methyl mustard oil	1:200,000	1: 600,000	1:200,000	1: 600,000	1:600,000	1:2,000,000
Ethyl mustard oil	1: 60,000	1: 200,000	1: 60,000	1: 60,000	1:200,000	1:2,000,000
Allyl mustard oil	1:200,000	1: 600,000	1:200,000	1: 600,000	1: 60,000	1: 600,000
Phenyl mustard oil	1: 60,000	1: 200,000	1: 60,000	1: 200,000	1: 30,000	1: 600,000
Benzyl mustard oil	1:600,000	1:6,000,000	1:600,000	1:2,000,000	1:200,000	1:2,000,000
Phenylethyl m. oil	1: 60,000	1: 600,000	--	1: 60,000	1: 60,000	1: 600,000

a = Limit concentration at which no bacterial growth occurs.

b = Concentration at which a distinct inhibition exists, compared with the control.

Table 2. Testing of bacteriostasis in the tube test.

Staph. aureus (SG 511)

a	b
1: 100,000	1:1,000,000
1: 30,000	1: 300,000
1: 30,000	1:1,000,000
1: 10,000	1: 30,000
1: 300,000	1:3,000,000
1: 300,000	1:3,000,000

a - Limit concentration at which clouding does not yet appear. b - Concentra tion at which a distinct difference from the control is visible.

Inhibition of human-pathogenic bacteria by benzyl mustard oil in the gas test (Table 3)

Table 3

		Typhoid	Paratyphoid	Diphtheria
Benzyl mustard oil	a)	1: 400,000	1: 400,000	1: 200,000
	b)	1:1,000,000	1:1,000,000	1:1,000,000

b = Concentration at which there is a distinct inhibition as aginst the contr

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The effectiveness against viruses was tested on the encephalitis virus Columb SH.

A mouse brain infected with this virus was admixed with benzyl mustard oil in the ratio A = 1:300,000, B = 1:1,000,000; C = control portion. After 24 h storag in the refrigerator, an equal number of mice was infected intracerebrally with t three solutions. While the animals of groups B and C died within the typical time, the animals of group A showed a clearly prolonged survival time, or respect ively they survived. The brains of all mice were subsequently incoluated on guinea pigs. Only the guinea pigs which had been inoculated with brains of grou A survived.

The mustard oils belong to the strongest skin irritants, hence their use as

external resolvents, i.e. for transfer onto the skin. They have a pungent, unpleasant odor and cause lacrimation. The toxicity after parenteral administrati was tested on white mice; solution in various concentrations in sperm oil; injection under the dorsal skin.

Shortly after the injection cerebral symptoms appeared, spasmic jumping about tonic cramps to the exitus, in the case of the easily volatile substances (methy and ethyl mustard oil) already after 5 minutes. If the animals survived the hou of spasmic fits, they died on the second day without striking symptoms. The toxicity is clearly dependent on the volatility of the mustard oil; phenylethyl mustard oil proved least toxic.

Table 4. Toxicity of the mustard oils after subcutaneous injection (*)

	Solution in sperm oil	LD_{50} (48 h) of the pure substance
Methyl mustard oil	1%	0.05 g/kg
Ethyl mustard oil	3.3%	0.11 g/kg
Allyl mustard oil	10%	0.08 g/kg
Phenyl mustard oil	25%	0.25 g/kg
Benzyl mustard oil	25%	0.15 g/kg
Phenylethyl mustard oil	25%	0.25 g/kg

(*) For embryonic tissue the toxicity of the mustard oils is much greater.

As local reaction there was found a heavy infiltrate, which usually ulcerated. Histological finding after 4 times half the LD_{50} of benzyl or phenylethyl mustard oil within 14 days (Dr. Berres, Histol. Lab. of the Skin Clinic, Cologne): At th point of injection the skin shows swelling, in the area of the inflammation or of an ulcer accumulated mitoses, all in the same stage, with agglomerated chromosomes. In the cutis: Cell nuclei with central vacuole, in part fractured nuclei The spleen shows hyperplasia of the white pulp, also the bone marrow is hyperplastic. The most striking modifications are found in the liver: Periportally marked round-cell infiltrates, the vessels are strikingly wide, the plasma of the liver cells shows a honeycomb type granular structure, the cell nuclei are regula

The kidneys and testicles are not substantially changed.

There was to be examined also the possibility of a serum inhibition of the bacteriostatic action. In some control experiments with blood agar plates, the benzyl mustard oil proved less active, so that in the gas test 3-10 times the quantity was necessary for the same inhibition. Similar results were obtained when fresh blood (1 cc) admixed with Na. citricum was placed next to or on the mustard oil. The blood seems to bind a considerable portion of the mustard oil.

The excretion of the mustard oil probably occurs in part through the kidney, as we were able to prove by our own tests in analogy to those of Winter with nasturtium (gas test: 30 cc of urine in the lower dish).

After 10 mg of benzyl mustard oil in milk or oil given by mouth, several of the urine portions eliminated between the second and tenth hours showed an inhibition on all test strains. Excretion occurs in varying concentration. Since the gas test corresponds to a considerable dilution of the active substance, we may assume for the urine a much stronger bacteriostatic action. None of the urine portions eliminated between the second and tenth hours showed bacterial decomposition even after incubation for 2 days.

To clarify the identity of the active substance of nasturtium with a mustard oil, an oily plant extract containing about 0.5% mustard oil (Winter (6)) was compared with a 0.5% oily solution of synthetic benzyl and phenylethyl mustard oil, respectively.

The results permit us with certainty to rule out the phenylethyl mustard oil as the active factor of the cress. In view of the low volatility and high absorption of the phenylethyl mustard oil to the sperm oil, it was 50-500 times less active in the gas test than the plant extract; especially with E. coli the difference from the plant extract is striking.

The spectrum of action and the elimination in the urine suggested at first th benzyl mustard oil, but also compared with it the plant extract was 6-30 times more active in the gas test. In the tube test, the benzyl mustard oil was 3 times less effective than the plant extract. Also when testing the urine, this difference is evident. Whether this factor occurring in the plant and after ad-

ministration of a galenic extract from the plant also in the urine, is synergist or additive to the mustard oils, we were unable to decide.

(For summary in English and literature references see p. 507).

(*) The work was done in the Chemical Laboratory of the Skin Clinic (headed by Prof. Dr. F. Schönhöfer). The research was carried out from the summer to fal of 1933.

Translated by Carl Demrick Associates, Inc./LH/db

[illegible]zität der Senföle bei subcutaner Injektion*)

	Lösung in Spermöl	DL_{50} (48 h) der Reinsubstanz
[illegible]ylsenfol	1%ig	0,05 g/kg
[illegible]l	3,3%ig	0,11 g/kg
[illegible]	10%ig	0,08 g/kg
[illegible]ylsenfol	27%ig	0,25 g/kg
[illegible]	27%ig	0,15 g/kg
[illegible]ylätylsenfol	27%ig	0,25 g/kg

[illegible] embryonales Gewebe ist die Toxizität der Senföle be[illegible].

[illegible]: Phenyläthylsenfol erwies sich als am schwäch[illegible].

Als [illegible] die Reaktion fand sich ein derbes Infiltrat, das [illegible]. Histologischer Befund nach 4mal je die [illegible] von Benzyl- oder Phenyläthylsenfol inner[illegible] 1½ Tagen (Dr. Berres, histol. Labor. der [illegible]nik, Köln): An der Injektionsstelle zeigt die Haut [illegible]erscheinungen, im Bereich der Entzündung [illegible] Ulcus gehäufte Mitosen, alle im gleichen Stadium, mit verklumpten Chromosomen. In der Cutis: Zell[illegible] mit zentraler Vakuole, z. T. frakturierte Kerne. [illegible] zeigt Hyperplasie der weißen Pulpa, auch das [illegible]mark ist sehr zellreich. Die auffälligsten Ver[illegible]rungen finden sich in der Leber: Periportal ausge[illegible] Rundzellinfiltrate, die Gefäße sind auffallend [illegible], das Plasma der Leberzellen zeigt eine wabig-kör[illegible] Struktur, die Zellkerne sind regelrecht. Nieren und [illegible] erscheinen nicht wesentlich verändert.

Zu untersuchen war auch die Möglichkeit einer Serum-[illegible]mmung der bakteriostatischen Wirkung. In einigen Kontrollversuchen mit Blutagarplatten erwies sich das Benzylsenfol als weniger wirksam, so daß im Gastest die 3—10fache Menge für die gleiche Hemmung notwendig war. Ähnliche Ergebnisse wurden gewonnen, wenn [illegible]ches, mit Na. citricum versetztes Blut (1 cm³) neben, bzw. auf das Senfol gegeben wurde. Das Blut scheint [illegible]n erheblichen Teil des Senfoles zu binden.

Die Ausscheidung des Senfoles erfolgt wohl z. T. über die Niere, wie nach Selbstversuchen analog den Winter'schen Versuchen mit der Kapuzinerkresse nachgewiesen werden konnte (Gastest: 30 cm³ Urin in die untere Schale).

Nach 10 mg Benzylsenfol in Milch oder Öl peroral zeigten mehrere der zwischen der 2. und 10. h abgegebenen Urinportionen eine Hemmung auf alle Teststämme. Die Ausscheidung erfolgt in wechselnder Konzentration. Da der Gastest einer erheblichen Verdünnung der aktiven Substanz entspricht, ist für den Harn eine viel größere bakteriostatische Wirkung anzunehmen. Keine der zwischen der 2. und 10. h gelassenen Harnportionen zeigte selbst bei 2tägigem Bebrüten bakterielle Zersetzung.

Zur Klärung der Identität des wirksamen Stoffes der Kapuzinerkresse mit einem Senfol wurde ein öliger Pflanzenextrakt mit ca. 0,5% Senfol (Winter [6]) mit einer 0,5%igen öligen Lösung von synthetischem Benzyl- bzw. Phenyläthylsenfol verglichen.

Die Ergebnisse lassen mit Sicherheit das Phenyläthylsenfol als den wirksamen Faktor der Kresse ausschließen. Bei der geringen Flüchtigkeit und großen Absorption des Phenyläthylsenfoles an das Spermöl war es im Gastest 50—500mal weniger wirksam als der Pflanzenextrakt, besonders bei *E. coli* fällt der Gegensatz zum Pflanzenauszug auf.

Das Wirkungsspektrum und die Ausscheidung im Harn sprachen zunächst für das Benzylsenfol, gegenüber dem aber auch im Gastest der Pflanzenauszug 6—30mal wirksamer war. Im Röhrchentest war das Benzylsenfol 3mal weniger wirksam als der Pflanzenauszug. Auch bei Testung des Urins wird dieser Unterschied deutlich. Ob dieser in der Pflanze und nach Verabreichung eines galenischen Extraktes aus der Pflanze auch im Urin auftretende Faktor synergistisch oder additiv zu den Senfölen wirkt, konnten wir nicht entscheiden.

Zusammenfassung:

Es wird über die bakteriostatische Wirkung einer Reihe von synthetisch hergestellten Senfölen (Methyl-, Äthyl-, Allyl-, Phenyl-, Benzyl-, Phenyläthylsenfol) berichtet. Bei den wirksamsten Verbindungen liegt die noch vollständig wachstumshemmende Grenzkonzentration etwa bei 1 : 600 000. Die Wirkung erstreckt sich auf eine große Anzahl grampositiver und -negativer Bakterien.

Die Toxizität der synthetisierten Senföle wurde an weißen Mäusen bei subcutaner Injektion bestimmt. Es ergab sich eine deutliche Abhängigkeit von der Flüchtigkeit des Öls: höchste Flüchtigkeit und Toxizität zeigte das Methylsenfol: DL_{50} = 0,05 g/kg; am wenigsten toxisch war das Phenyläthylsenfol: DL_{50} = 0,25 g/kg.

Serum bzw. Blut setzen die bakteriostatische Wirkung des Benzylsenfols im Plattenversuch um den Faktor 3—10 herab.

Die Ausscheidung erfolgt z. T. über die Niere. Nach Einnahme von 10 mg Benzylsenfol ist der Harn länger als 10 h bakteriostatisch wirksam.

Die Pflanzenextrakte aus *Tropaeolum maius* L. waren bedeutend wirksamer als jedes der untersuchten Senföle.

Wir haben Herrn Prof. Dr. phil. habil. G. Winter, dem Leiter der botanischen Abteilung und der Firma Dr. Madaus, Köln, für die Unterstützung zu danken. Der galenische Extrakt aus *Tropaeolum* ist unter dem Markenzeichen „Tromalyt" eingeführt.

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Summary:

Investigations of the Bacteriostatic Action of some Mustard Oils

Report on the bacteriostatic action of some synthetic mustard oils (methyl, ethyl, allyl, phenyl, benzyl and phenylethyl mustard oil). In the most effective compounds, the limiting value of full activity is about 1:600000. The action covers a large number of grampositive and gramnegative bacteria.

The toxicity of synthetic mustard oils was determined by subcutaneous injection in white mice. The toxicity appears to depend largely on the degree of volatility. Methyl mustard oil is the most volatile and most toxic agent, the DL_{50} is 0,05 gs per kg body weight. Phenylethyl mustard oil is the least toxic: DL_{50} = 0,25 gs per kg.

Plate tests showed that serum or blood reduce the bacteriostatic action of benzyl mustard oil 3—10-fold.

Mustard oils are partly excreted by the kidneys. After application of 10 mgs benzyl mustard oil the urine will show bacteriostatic action for more than 10 hrs.

Plant extracts from *Tropaeolum maius* proved considerably more effective than any of the mustard oils.

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Aus der Forschungs-Abteilung der Austria Pan-Chemie, Wolfsberg in Kärnten (Österreich)

Chemie und Galenik der Usninsäure

Von Dr. habil. H. von Czetsch-Lindenwald

Waksman definierte folgendermaßen: „Ein Antibiotikum ist eine chemische Substanz, die von Mikroorganismen erzeugt wird und die Fähigkeit besitzt, Bakterien und andere Mikroorganismen zu zerstören." Im Laufe der Jahre kamen zu den wirksamen Stoffen aus Mikroorganismen auch solche aus höheren Pflanzen, ja sogar Tieren, so daß der Teil der Definition, der als Erzeuger von Antibioticis nur Mikroorganismen anführt,

(German publication, pp. 1374-6)

Effect of the "essential" phospholipids on hepatic lesions after chronic intoxication with ethyl alcohol and allyl isosulfocyanate in white rats

By Leonidas Samochowiec, Jerzy Wojcicki and Konstanty Dominiczak

The pathogenesis of cirrhosis of the liver is not yet fully understood. Because of its direct or indirect effect on liver cells, ethyl alcohol is unquestionably of some importance in the development of hepatic lesions, but other exogenic hepatoxic factors were to be taken into account as well. Interesting, for example, is the clinical observation of Lohman (10) that in children who had been given considerable amounts of mustard over a prolonged time severe liver-spleen syndromes with symptoms of cirrhosis of the liver occurred. Mustard being a readily available condiment, it is particularly fancied as a relish by persons who drink much alcohol.

One of the constituents of table mustard is black pepper. It contains the glucoside sinigrin, which is degraded by the simultaneously occurring enzyme myrosin and is hydrolyzed to allyl isosulfocyanate (15). The latter is referred to in this paper by the abbreviation AIS.

In the present experiment, rats were fed with alcohol and AIS and the attempt was made to treat the lesions caused by such intoxication with "essential" phospholipids (abbreviated EPL) (*).

The "essential" phospholipids represent a biologically highly active form of the combination of polyenic acids with phosphatidyl choline. As biological emulsifiers they have a major share in the transport, dissolution, and utilization of the lipids in the liver and elsewhere (13), as was demonstrated by experiment and clinical observation (1, 3, 18).

Material and method

The tests were carried out on 60 white male Wistar rats of the same age, weighing between 200 and 280 g.

The animals were divided into 6 groups of equal number, as follows:

Group I: Control animals

Group II: The animals were given in the morning, on fasting, 20% ethanol orally in a dosage of about 1.5 ml/100 g body weight and 0.1 ml 5% glucose solution i.p.

Group III: The animals were given about 0.002 mg AIS/100 g body weight orally with the drinking water and 0.1 ml 5% glucose solution per 100 g, i.p

Group IV: The animals were given simultaneously AIS and alcohol orally in the dosage as in Group III and II and 0.1 ml 5% glucose solution per 100 g, i.p.

Group V: The animals were given AIS as in Group III plus EPL in a 5% glucose solution (1:7) i.p. in a dosage of 0.7 mg/100 g body weight.

Group VI: The animals were given AIS and alcohol as in Group IV and EPL as in Group V.

As basic diet a granulated feed was given in the composition evolved at the Warsaw Institute of Medicaments, as well as about 10 ml of milk and water ad libitum. Before being killed, the animals did not eat for 24 h and were given no alcohol. The experiment took 70 days.

The following serum examinations were carried out: Test for esterified fatty acid content by the hydroxam method in the modification of Morgan & Kingsbury (11). Total cholesterol content in analogy to the reaction according to Lifschültz and the thymol turbidity test according to the manual of Tulczynski (16).

The blood samples for the biochemical tests were taken after opening of the thorax under light ether narcosis from the ascending part of the aorta. For the histological tests liver sections were fixed in 10% neutralized formalin solution, washed, and sections 12 to 15 microns thick were prepared in the freezing microtome. Staining was done in Oil Red, Sudan III, and with hematoxylin and eosin. The numerical results were examined statistically by the Student t-test.

Results

In the serum of the animals which had received only alcohol, only AIS, or alcohol plus AIS, the content of esterified fatty acids was clearly increased over the controls (Table 1). The rise ranged between 234 and 417% and was statistically significant. The greatest increase appeared in Group III, which

had received only AIS. In animals which had been given AIS + EPL or AIS + alcoho + EPL, the values of the esterified fatty acids were considerably below the valu of the untreated groups, but did not reach the values of the control group.

Table 1. Esterified fatty acids in the serum (mEg/l). *) + Mean standard deviation; a Alcohol; AIS Allyl isosulfocyanate; EPL "Essential" phospholipids.

Group	No. of animals	Substance given	Mean value *)	Rise (+) or inhibition (-) of rise in %	P
I	10	-	0.90 ± 0.12		
II	8	A	3.51 ± 0.62	1/2 + 290	1/2 < 0.001
III	9	AIS	3.65 ± 0.40	1/3 + 417	1/3 < 0.001
IV	8	AIS + A	3.01 ± 0.58	1/4 + 234	1/4 < 0.01
				2/4 - 14	2/3 > 0.040
V	9	AIS + EPL	1.45 ± 0.16	1/5 ± 61	1/5 < 0.05
				3/5 - 69	3/5 < 0.001
VI	8	AIS + A + EPL	1.92 ± 0.18	1/6 + 113	1/6 < 0.001
				4/6 - 36	4/6 > 0.10

Also the serum cholesterol level (Table 2) showed a distinct rise in Groups II, III and IV, namely about double that in the control group. Also these differences were statistically established. In the serum of the animals treated with EPL, the cholesterol values did not differ much from the norm.

Table 2. Serum cholesterol values (in mg%). For explanations see Table 1

Group	No. of animals	Substance given	Mean Value *)	Rise (+) or inhibition (-) of rise in %	P
I	10	-	42.4 ± 6.6		
II	10	A	84.4 ± 8.9	1/2 + 99	1/2 < 0.01
III	9	AIS	100.0 ± 5.3	1/3 + 135	1/3 < 0.001
IV	8	AIS+A	92.7 ± 14.9	1/4 + 129 2/4 + 10	1/4 < 0.01 2/4 > 0.60
V	9	AIS + EPL	50.6 ± 5.9	1/5 + 19 3/5 - 50	1/5 > 0.20 3/5 < 0.001
VI	10	AIS + A + EPL	48.4 ± 7.3	1/6 + 14 4/6 - 48	1/6 > 0.40 4/6 < 0.05

In the thymol turbidity test (Table 3) the sera showed a significant increase of the turbidty after chronic alcohol and AIS administration. This was clearest in the group which had received alcohol and AIS simultaneously.

The additional administration of EPL in groups V and VI resulted in a noticeably reduced turbidity, although also here the value determined for the control group was not reached.

Table 3. Results of the thymol turbidity test (MacLagan units). For explanations see Table 1.

Group	No. of animals	Substance given	Mean value *)	Rise (+) or inhibition (-) of rise in %	P
I	8	-	0.40 ± 0.05		
II	9	A	1.31 ± 0.14	1/2 + 227	1/2 < 0.001
III	7	AIS	1.99 ± 0.15	1/3 + 397	1/3 < 0.001
IV	8	AIS + A	2.35 ± 0.40	1/4 + 487 2/4 + 79	1/4 < 0.001 2/4 < 0.05
V	8	AIS + EPL	0.77 ± 0.17	1/5 + 92 3/5 - 61	1/5 > 0.05 3/5 < 0.001
VI	8	AIS + A + EPL	0.73 ± 0.10	1/6 + 82 4/6 - 69	1/6 < 0.05 4/6 < 0.01

The morphological changes appeared as fat inclusions in the cytoplasm (Table

4). In the control group they were slight and detectable only in occasional cells. Administration of alcohol, AIS or AIS + alcohol led to an increase in the number and size of the fat drops in Group II; in Group III double, and in Group IV even triple, the quantity of fat infiltrates was found (Table 4). The small fat drops merged to form depositions where the drops lay so close together, in Group IV, that sometimes the entire cell was filled with them. The histological picture was unusually clear in Group VI, which had received EPL in addition to AIS and alcohol. No fat inclusions were found in the liver cells of any animal of this group. For unexplained reasons Group V (AIS + EPL) showed an inconsistent picture.

Table 4. Results of the histological liver examinations. + - : Degree of liver cell adiposis; for other explanations see Table 1.

Group	Substance given	Staining	Liver cell adiposis					
I	-	Oil Red	+ -	+ -				
		Sudan	-	-				
II	A	Oil Red	+ -	+	+ -	+ +		
		Sudan	-	-	+	+		
III	AIS	Oil red	+	+++	+ +	+ +		
		Sudan	+ -	+ +	-	+		
IV	AIS + A	Oil Red	+++	++++	+ +	+ +	+++	+++
		Sudan	+++	+++	+++	+	+ +	+ +
V	AIS - EPL	Oil Red	+	++++	+ -	++++	-	
		Sudan	-	++++	-	+ +	-	
VI	AIS - A - EPL	Oil Red	-	-	-	-		
		Sudan	-	-	-	-		

Discussion of the results

As the above tests on white rats show, alcohol and AIS, respectively, bring about a distinct rise of the esterified fatty acids and of the cholesterol in the serum, an increase of the turbidity in the thymol turbidity test, and an

adiposis of the liver cells. After simultaneous administration of alcohol and AIS, however, no noticeable increase of the fatty acid and cholesterol content i seen. But the thymol turbidity test and the liver cell adiposis are then more pronounced than under the influence of alcohol or AIS alone.

Ethanol causes fatty changes in the liver (2, 5, 8, 9), but the mechanism of this degenerative liver cell damage is not yet fully understood. In the opinion of some authors, the fat infiltration of the liver is a result of the fatty acid synthesis stimulated by the alcohol. Others attribute greater importance to the mobilization of fat from the subcutaneous tissue. At the same time an increased cholesterol level in the serum is found (4, 6). We were interested in the effec of AIS on the genesis of such alterations because what is here concerned is a constituent of table mustard, which is a widely used condiment.

The thymol test, and more clearly still the histological examinations of the liver, showed indeed that adiposis of this organ can be brought about by AIS alo and by alcohol alone, but that fatty degeneration is seen most clearly when these two compounds are administered simultaneously.

A comparison of the histological finding with the results of the biochemical serum determinations seems to justify the assumption that the deviations of the biochemical serum values reflect in high degree the changes that have occurred in the liver, the more so as the liver plays an important part in the lipid and cholesterol metabolism. The thymol turbidity test is also of great diagnostic importance in liver damage (14), though it is not specific for the diseases of this organ.

Now if we take into consideration, on the one hand, the importance of the "essential" phospholipids for the general fat metabolism of the body and, on the other, the reports from the literature on the inhibiting effect of alcohol on the phospholipid synthesis in the liver (7, 17), it appeared logical to undertake an attempt of therapy with EPL in lesions caused experimentally by long-term administration of ethanol and AIS. The results of the biochemical serum examinations and the histological findings have justified this attempt.

Conclusions

1. After chronic administration of ethanol and allyl isosulfocyanate there occurs an increase of the esterified fatty acids and of the cholesterol level in the serum, and the results in the thymol turbidity test are highly positive.

2. Histologically, the most distinct degenerative changes were found in the livers of the animals which had received alcohol and AIS simultaneously.

3. "Essential" phospholipis protect against development of the above describ changes. The protective effect is reflected in the biochemical picture of the serum as well as in the histological finding of the liver.

(For summary and literature references see p. 1376).

(*) We are indebted to A. Nattermann & Cie., Köln-Braunsfeld, for the EPL substance made available to us.

Translated by Carl Demrick Associates, Inc./LH/db

Besprechung der Ergebnisse

Wie die obigen Versuche an weißen Ratten zeigen, bewirken Alkohol bzw. AIS einen deutlichen Anstieg der veresterten Fettsäuren und des Cholesterins im Serum, eine Zunahme der Trübung im Thymoltrübungstest und eine Verfettung der Leberzellen. Nach gleichzeitiger Verabreichung von Alkohol und AIS läßt sich dagegen kein merklicher Anstieg des Fettsäuren- und Cholesteringehaltes feststellen; der Thymoltrübungstest und die Leberzellverfettung sind aber hier deutlicher ausgeprägt als unter dem Einfluß von Alkohol bzw. AIS allein.

Athanol ruft in der Leber fettige Veränderungen hervor [2, 5, 8, 9], doch ist der Mechanismus dieser degenerativen Leberzellschädigung noch nicht ganz geklärt. Nach Ansicht verschiedener Autoren ist die fettige Infiltration der Leber eine Folge der durch den Alkohol stimulierten Fettsäuresynthese. Andere dagegen messen der Fettmobilisierung aus dem subcutanen Gewebe größere Bedeutung zu. Gleichzeitig wird ein erhöhter Cholesterinspiegel im Serum festgestellt [4, 6]. Uns interessierte der Einfluß von AIS auf die Entstehung solcher Veränderungen, denn es handelt sich hierbei um einen Bestandteil des Speisesenfs, der eine weit verbreitete Speisewürze darstellt.

Tatsächlich ergaben die Thymolprobe und noch deutlicher die histologischen Untersuchungen der Leber, daß zwar AIS allein und Alkohol allein eine Verfettung dieses Organs bewirken können, daß aber das deutlichste Bild einer fettigen Degeneration unter der gleichzeitigen Verabreichung dieser beiden Verbindungen entsteht.

Ein Vergleich des histologischen Befundes mit den Ergebnissen der biochemischen Serumbestimmungen scheint die Annahme zu rechtfertigen, daß die Abweichungen der biochemischen Serumwerte in hohem Maße die in der Leber vor sich gegangenen Veränderungen wiederspiegeln, dies um so mehr, als die Leber eine wichtige Rolle im Lipid- und Cholesterinstoffwechsel spielt. Der Thymoltrübungstest ist ebenfalls von großer diagnostischer Bedeutung bei Leberschäden [14], obgleich er für die Erkrankungen dieses Organs nicht spezifisch ist.

Zieht man nun einerseits die Bedeutung der „essentiellen" Phospholipide für den allgemeinen Fettstoffwechsel des Organismus und andererseits die Mitteilungen aus dem Schrifttum über die hemmende Wirkung des Alkohols auf die Phospholipidsynthese in der Leber [7, 17] in Betracht, so erschien es sinnvoll, einen Therapieversuch mit EPL bei experimentell durch langzeitige Verabreichung von Athanol und AIS hervorgerufenen Schäden zu unternehmen. Die erzielten Resultate der biochemischen Serumuntersuchungen und die histologischen Befunde rechtfertigen den unternommenen Versuch.

Schlußfolgerungen

1. Nach chronischer Verabreichung von Athanol und Allylisosulfocyanat kommt es zu einem Anstieg der veresterten Fettsäuren und des Cholesterinspiegels im Serum und zu stark positiven Ergebnissen im Thymoltrübungstest.

2. Histologisch wurden die deutlichsten degenerativen Veränderungen in den Lebern der Tiere gefunden, die gleichzeitig Alkohol und AIS erhalten haben.

3. „Essentielle" Phospholipide schützen vor der Entstehung der oben beschriebenen Veränderungen. Die Schutzwirkung findet im biochemischen Bild des Serums wie in dem histologischen Befund der Leber einen Ausdruck.

Zusammenfassung

Im Tierversuch an weißen Ratten wurde der Einfluß von „essentiellen" Phospholipiden (EPL) auf die unter langzeitiger Verabreichung von Athanol und Allylisosulfocyanat (AIS) entstehenden Veränderungen untersucht.

Es wurden im Serum die gesamten veresterten Fettsäuren und das Cholesterin bestimmt, der Thymoltrübungstest durchgeführt und die Leber histologisch untersucht.

Die Serumkontrollen ergaben eine Zunahme der veresterten Fettsäuren und des Cholesterins und einen stark positiven Thymoltrübungstest; histologisch wurde eine Leberverfettung nachgewiesen, die bei gleichzeitiger Alkohol- und AIS-Verabreichung am ausgeprägtesten war.

Die „essentiellen" Phospholipide (Polyensäuren + Phosphatidylcholin) verhindern solche Veränderungen, wie durch biochemische Untersuchungen des Serums und histologische Kontrolle der Leber bewiesen wurde.

Summary

The Influence of the „Essential" Phospholipids on Hepatic Lesions Caused by Chronic Administration of Ethanol and Allylisosulfocyanate

In white rats the influence of "essential" phospholipids (EPL) on the changes caused by chronic administration of ethanol and allylisosulfocyanate (AIS) was studied.

The total esterified fatty acids and cholesterin in the serum were determined, the thymol turbidity test was performed, and the liver was examined histologically.

The serum controls revealed an increase of the esterified fatty acids and cholesterin and a highly positive turbidity test; histologically fatty degeneration of the liver was proved, which was most marked after combined administration of alcohol and AIS.

The "essential" phospholipids (polyenic acids + phosphatidyl choline) prevent such changes as proved by biochemical studies of the serum and histological examination of the liver.

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MUSTARD SEED OIL MEAL AND SUNFLOWER SEED OIL MEAL IN CHICK STARTER RATIONS[1]

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Because of the present day shortage of animal proteins for poultry rations, the use of proteins from vegetable sources has become more widespread. These latter have been used to replace varying amounts of the individual or a combination of the animal proteins. Several of the newer vegetable proteins are sunflower seed oil meal and mustard seed oil meal. Pettit *et al.* (1) reported that under the conditions of their experiment, sunflower seed oil meal, when replaced on a protein equivalent basis, could be used as a complete substitute for meat meal in chick starters. As far as the author is aware, no information is available on the use of mustard seed oil meal as a protein supplement in chick starter rations.

An experiment was undertaken to compare mustard seed oil meal and sunflower seed oil meal and to add further information on the value of sunflower seed oil meal as a partial substitute for animal proteins.

MATERIAL AND METHODS

Eight randomized groups of 40 unsexed day-old New Hampshire chicks were used in this test. Group 1 constituted the control group (no vegetable protein), groups 2-4 and 5-8 were the experimental lots which were fed increasing amounts of sunflower seed oil meal and mustard seed oil meal, respectively (see Table 3). When one day of age, the chicks were weighed individually and each group was placed in a separate compartment of an electric battery brooder. At two weeks of age, the chicks were transferred to floor pens. Body weight was recorded bi-weekly for the eight weeks of the test. Feed and water were supplied *ad lib.*

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TABLE 1.—COMPOSITION OF THE BASAL MASH

Ingredients	Lb.
Ground wheat	37
Ground oats	20
Ground barley	17
Bran	5
Fish meal	2½
Meat meal (55%)	3½
Buttermilk powder	2
Vitagras	2
Alfalfa meal	1½
Limestone powder	½
Salt	½
Fish oil (1200A-200D)	½
Manganese sulphate	¼ lb. per ton
Riboflavin premix	.....*
Total	92

* Sufficient added to supply 1700 micrograms of riboflavin per pound of feed.

TABLE 2.—CHEMICAL ANALYSIS OF THE BASAL DIET AND SUPPLEMENTS

Supplement	Moisture	Protein	Ash	Ca.	P_2O_5
	%	%	%	%	%
Basal diet	7.7	19.5	4.96	0.80	1.37
Animal protein	6.7	52.3	16.37	4.66	5.95
Sunflower seed oil meal	6.3	37.4	6.92	0.44	2.84
Mustard seed oil meal	6.9	37.4	5.63	0.55	2.04

TABLE 3.—AMOUNTS OF SUPPLEMENTS ADDED TO THE BASAL DIET

Diet and group	1	2	3	4	5	6	7	8
	lb.	lb.	lb.	lb.	lb.	lb.	lb.	lb.
Basal diet	92.0	92.0	92.0	92.0	92.0	92.0	92.0	92.0
Animal protein	8.0	6.0	3.0	—	6.0	4.0	2.0	—
Sunflower seed oil meal	—	2.8	7.0	11.2	—	—	—	—
Mustard seed oil meal	—	—	—	—	2.8	5.6	8.4	11.2

The basal mash as well as the supplements were analysed for protein, calcium, phosphorus and ash. In order to maintain the same protein level in all groups of chicks, it was necessary to substitute greater amounts of the vegetable proteins because of their lower protein content. No other adjustment was made at this time since the relatively small amounts added would not materially affect the balance of the ration.

The basal ration is listed in Table 1.

From the above table it will be seen that the animal protein is in the proportion of 2½, 3½ and 2 for the fish meal, meat meal and buttermilk powder, respectively. When adding additional animal protein to the basal mash of the various groups, the above proportion was maintained. Table 2 presents the chemical analysis of the basal diet along with the supplements.

In order to maintain the same protein level in the mash of all groups of chicks, 1.4 pounds of vegetable protein was substituted for 1.0 pound of the animal protein mixture. Table 3 outlines the method of substitution.

DISCUSSION OF RESULTS

The unweighted means of body weight on a bi-weekly basis from hatching time to eight weeks of age are listed in Table 4. An examination of the original data indicated that the average body weight of the various groups was not affected by sex, since there was no significant difference in the ratio of males to females within each group. A comparison of the body weights of those groups on the different supplements indicates that mustard seed oil meal as fed under the conditions of this experiment is not a good substitute for animal protein. On the other hand, those groups receiving sunflower seed oil meal were quite comparable to the control lot. Table 5 summarizes the analysis of the weight data at eight weeks of age. The method of analysis employed was that of "Weighted squares of means" as outlined by Yates (3) and Titus and Hammond (2).

TABLE 4.—UNWEIGHTED MEANS OF BODY WEIGHT

Group	Age in weeks				
	0	2	4	6	8
	gm.	gm.	gm.	gm.	gm.
1	35.81	125.09	315.04	597.27	892.08
2	36.02	116.29	318.55	606.90	893.13
3	36.05	113.80	297.05	553.11	881.37
4	36.31	110.29	291.37	557.66	857.41
5	35.90	109.60	286.85	552.74	825.02
6	36.37	114.22	290.35	550.55	846.66
7	35.58	111.00	285.50	538.17	833.24
8	36.95	110.00	289.11	526.36	803.92

TABLE 5.—TESTS OF SIGNIFICANCE BETWEEN DIETS

Group	2	3	4	5	6	7	8
			%	%	%	%	%
1 vs.	†	—	5*	1**	1	1	1
2 vs.		—	5	1	1	1	1
3 vs.			—	—	5	1	1
4 vs.				5	—	—	1
5 vs.					—	—	—
6 vs.						—	1
7 vs.							—

† No significant difference.
* Significant at 5% point.
** Significant at 1% point

Groups 2 and 3 did not differ significantly in average final body weight when compared to the control group. Group 4 (basal plus 11.2 per cent sunflower seed oil meal) was significantly lower in weight than Group 1. This is not in agreement with Pettit *et al.* (loc. cit.) who found that up to 14 per cent of the sunflower seed oil meal, when substituted on an equivalent protein basis, could be used to replace meat meal. This represented a complete substitution for the meat meal. However, it should be pointed out that these authors fed bone meal and oyster shell *ad lib.* in addition to 1 per cent of each of these ingredients in the basal mixture. Our basal diet contained only ½ per cent limestone powder and no bone meal. Reference to Table 2 indicates that the vegetable protein feeds are lower in both calcium and phosphorous than the animal protein mixture. It is not possible to state whether one or both of these minerals, if fed in greater amounts, would account for the differences noted herein. However, when not more than one-third of the total animal protein is replaced by sunflower seed oil meal, growth is satisfactory.

With respect to the groups receiving the mustard seed oil meal, the above statement may partly account for the significantly lower weights. Group 5 which received only 2.8 per cent mustard seed meal as well as 6 per cent of the animal protein mixture in the basal diet was significantly lower in body weight than either the control lot or Group 2 (2.8 per cent

sunflower seed oil meal plus 6 per cent animal protein). As the mineral content of both supplements was similar, the significantly lower weight cannot be explained entirely on this basis. Mustard seed oil meal as a feed ingredient is not unpleasant to taste (from the human standpoint) since the bitter taste of the mustard seed is removed with the extraction of the oil. No differences were noted in the feed consumption figures from the various groups; this would eliminate the possibility of the taste of the mustard seed oil meal being a factor which might account for the lowered rate of growth. No abnormalities were observed in any of the groups except one bird in Group 8 which developed perosis.

Mortality was very low throughout the test. The total loss of chicks was four—one from Group 1; one from Group 3, and two from Group 7.

SUMMARY

A basal diet containing 19.5 per cent protein was supplemented with varying amounts of either mustard seed oil meal or sunflower seed oil meal and fed to growing chicks. The results obtained warrant the following conclusions:

1. Sunflower seed oil meal is a satisfactory substitute in chick starters when replacing not more than one-third of the total animal protein mixture.

2. Mustard seed oil meal is comparable to sunflower seed oil meal when analysed for protein, calcium and phosphorus.

3. The substitution of mustard seed oil meal in quantities as small as 2.8 per cent (equivalent to 2 per cent animal protein mixture) results in significantly lower body weight at eight weeks of age.

4. The poor growth resulting from the feeding of mustard seed oil meal cannot be explained fully on the basis of its calcium and phosphorus content.

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Research Section

Food Flavourings and Compounds of Related Structure. II. Subacute and Chronic Toxicity

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Abstract—The subacute and/or chronic toxicity of 48 food flavourings was studied in rats. The toxicity of five of these flavourings (coumarin, 6-methylcoumarin, dihydrocoumarin, methyl salicylate and safrole) was also studied in dogs. A summary of the studies is presented. The loss of 21 flavourings from laboratory animal diets during a 7-day period was also determined.

INTRODUCTION

For a number of years the Food and Drug Administration has been investigating the toxicity of food flavourings, both natural and synthetic. The toxicity studies consist of (1) determination of the acute oral effects, (2) subacute studies in which the flavouring agents are mixed in the diet or administered by stomach tube, and (3) chronic feeding studies on those flavouring agents having wide use, having chemical structures implying toxicity and/or showing toxic effects in the subacute studies. The data on the acute oral toxicity of these substances have been reported (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The present report is a summary of the subacute and chronic studies. Full reports on some of the chronic studies have been published for methyl salicylate (Webb & Hansen, 1963), safrole (Long, Nelson, Fitzhugh & Hansen, 1963; Hagan, Jenner, Jones, Fitzhugh, Brouwer & Webb, 1965) and dihydrosafrole (Long & Jenner, 1963; Hagan *et al.* 1965).

Commercially-available materials, rather than pure chemicals, were used since the purpose of these studies was to evaluate the toxicity of these materials in relation to their use as food additives.

EXPERIMENTAL

Rat studies. Weanling Osborne–Mendel rats were used in the chronic and most of the subacute experiments. In a few subacute experiments young adult rats of the same strain were used. Litter mates were used except for a few subacute studies in which the weanlings were randomized by weight (every level having animals of equal weight). A control group containing the same number of rats as the test groups was included with each test compound except in the following studies in which a common group was used for a number of test compounds:

(1) Allyl caproate, allyl cyclohexane propionate, anisaldehyde, benzaldehyde, carvone, dimethylbenzyl carbinol, ethyl sebacate, geraniol extra, piperonal and vanillin fed at 1000 ppm for 27–28 wk (control group 10 males and 10 females).

(2) Anethole, anisaldehyde, benzaldehyde, carvone, dimethylbenzyl carbinol, Dolcourin, ethyl methylphenyl glycidate, ethyl pelargonate, ethyl sebacate, geraniol extra, isoeugenol, piperonal and vanillin fed at 10,000 ppm for 16 wk (control group 10 males and 10 females).

(3) Benzyl cinnamate, eugenol and thymol fed at 1000 and 10,000 ppm for 19 wk (control group 5 males and 5 females).

(4) Anethole, carvone and ethyl methylphenyl glycidate fed at 2500 ppm for 1 yr (control group 5 males and 5 females).

(5) Allyl caproate and allyl cyclohexane propionate fed at 2500 ppm for 1 yr (control group 5 males and 5 females).

(6) Ethyl vanillin and vanillin fed at 5000, 10,000 and 20,000 ppm for 2 yr (control group 12 males and 12 females).

(7) Ethyl vanillin and vanillin fed at 20,000 and 50,000 ppm for 1 yr (control group 5 males).

(8) Dihydrosafrole, isosafrole and safrole fed at 1000, 2500, 5000 and 10,000 ppm for 2 yr (control group 35 males and 35 females).

The animals were housed individually in wire cages and had access to food and water at all times. Most of the compounds were fed in the diet, but a few were administered by stomach tube. Fresh diets were made and distributed weekly except in the study with allyl isothiocyanate in which the diet was prepared and fed daily*. When the compound was administered by stomach tube, corn oil solutions of the flavouring were prepared. The concentration of the solution was adjusted for each level so that all rats received a constant volume of 1 ml of solution/kg daily. The control animals were given an equal volume of corn oil. The rat's weight, food intake and general condition were recorded every week. Haematological examinations were made at termination of the subacute studies, and at 3, 6, 12 and 22 months in the chronic experiments. These examinations included white cell counts, red cell counts, haemoglobins and haematocrits.

At the termination of the experiments the rats were sacrificed and exsanguinated. The tissues of all the rats were examined macroscopically at the time of sacrifice. The viscera were removed and the liver, kidneys, spleen, heart, and testes were weighed. These organs, the remaining abdominal and thoracic viscera, and one hind leg, for bone, bone marrow, and muscle, were preserved in 10% buffered formalin-saline solution for histopathological examination. For routine histopathology, sections were embedded in paraffin wax and stained with haematoxylin and eosin. Tissues from rats dying during the experiment were examined for gross changes and were preserved if autolysis was not advanced. Organs were not weighed but abnormalities and the suspected reason for death were noted.

Detailed microscopic examinations in the subacute studies were generally done on 6 or 8 rats, evenly divided by sex, from the high dose group and the control group. If changes attributable to the test compound were found in the high dose group, additional animals on lower dosage levels were examined as indicated. For animals from the long-term studies, more extensive histopathological study was done.

Dog studies. Pure-bred beagles were used in the studies with safrole and methyl salicylate. Mongrels and beagles were used in the coumarin, dihydrocoumarin, and 6-methylcoumarin studies. The dogs were wormed and immunized against distemper, rabies, and infectious

*Two other exceptions were the safrole rat study (Long *et al.* 1963) and the chronic rat feeding study of methyl salicylate (Webb & Hansen, 1963) in which the diets were prepared every other week and stored under refrigeration in sealed containers.

hepatitis before treatment was begun. They were housed in individual cages with food (commercial dry dog food) and water available at all times. The test compound was administered by capsule daily for 6 days a week. The animals were weighed weekly and the dosages were recalculated at this time. Haematological examinations were made three times prior to the start of treatment and at 2 wk, 1 month, 3 months, 6 months and 1 yr after treatment began and then at yearly intervals until termination of the study.

At the end of a study, survivors were killed with an overdose of an intravenous barbiturate and exsanguinated. The major organs were weighed. Sections of brain (four levels), pituitary, salivary gland, thyroid, parathyroid, thymus, lymph nodes, lung, heart, stomach, spleen, pancreas, kidney, adrenal, liver, gall bladder, small intestine (three levels), large intestine, urinary bladder, rib, skeletal muscle, and testis and prostate (or ovary and uterus) were preserved in 10% buffered formalin–saline for histological examination. In addition liver, kidney and adrenal sections and a femoral marrow plug were fixed in Zenker's solution. The sections were embedded in paraffin wax and stained with haematoxylin and eosin. Frozen sections of the liver and kidney were stained with Oil Red O for fat. A rib marrow smear was air-dried and stained with the Wright–Giemsa stain. Dogs found in a moribund condition during the experiment were sacrificed and necropsied. Dogs dying during the experiment were necropsied and tissue sections preserved if autolysis was not too advanced. All the tissues that were preserved were examined microscopically.

RESULTS

Table 1 shows the compounds tested; the dose levels; number, sex and species of test animals; duration; and effects. When the dose level is given in ppm, the test compound was mixed in the diet. When the dose is given in mg/kg, the test compound was administered daily by stomach tube to rats and (6 days/wk) by capsule to dogs. Only those effects believed to be due to the administration of the compound are listed. The designation "no effect" indicates that there was no effect on growth or haematology, and no macroscopic or microscopic change in the tissues. The designation "no macroscopic effect" indicates that there was no effect on growth or haematology, and no macroscopic change in the tissues, and that microscopic examination of the tissues was not performed. Pathological changes attributable to disease or age have not been included. For those compounds on which the toxicity studies have been reported previously the reference is given in Table 1 with the name of the compound.

In the evaluation of the data from the feeding studies with flavouring agents, the possibility of loss of the test compound from the experimental diet must be considered. Many of the flavouring agents used in these studies were quite volatile. Studies in our laboratory have shown that a decrease in the concentration of some of the flavourings occurred when the experimental diet was exposed in the open feed cups (Jones, Taylor, & Hagan, 1962). In order to determine the actual amount of the flavouring consumed by the animals, many of the experimental diets were analysed for the flavouring immediately after preparation and again after being exposed to laboratory conditions for 7 days. The flavourings on which analytical studies have been made and the percentage loss calculated over a 7-day period are presented in Table 2.

O = Not in FEMA GRAS III

Table 1. *Summary of subacute and chronic toxicity of food flavourings and related compounds*

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Acetophenone (methyl 1-phenyl ketone)	✓ 10,000	Rat 10M & 10F	17 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Allyl butyrate (allyl butanoate)	90 mg/kg	do.	18 wk	Growth retardation in males. Liver: macroscopic—rough or granular surface, firm consistency, nutmeg appearance; microscopic—slight to moderate bile-duct proliferation and fibrosis with pseudolobule formation. Necrosis with polymorphonuclear infiltration and swollen, foamy liver cells in 2 of 8 rats examined
	50 mg/kg	do.	17 wk	Lung—slight to marked peribronchial lymphocytic infiltration[b]. Liver—no effect
Allyl caproate (allyl hexanoate)	✓ 2500	5M & 5F	1 yr	No effect
	1000	do.	28 wk	do.
	100 mg/kg	10M & 10F	18 wk	Liver: macroscopic — somewhat nodular and wrinkled with granular or rough surface; microscopic—slight to moderate bile-duct proliferation, some lobular architectural disarrangement, slight fibrosis, pigment in macrophages; necrotic foci in 2 of 8 rats examined
	65 mg/kg	do.	do.	Liver: microscopic—very slight bile-duct proliferation in 2 of 8 rats examined[c]
	15 mg/kg	do.	do.	No effect[c]
Allyl cyclohexane propionate (allyl 3-cyclohexylpropionate)	✓ 2500	5M & 5F	1 yr	No effect
	1000	do.	27–28 wk	do.
Allyl isothiocyanate[d]	✓ 10,000	do.	26 wk	do.
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.

[a]Unless otherwise stated.
[b]Controls had slight to marked peribronchial lymphocytic infiltration not as severe as with the 50 mg/kg/day dose.
[c]Microscopic examinations were made on the liver only.
[d]The diet was prepared daily because of the extreme volatility of this compound. A control group pair-fed with the 10,000 ppm group was included in this study in addition to the usual untreated control group.

Table 1 continued

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Allyl isothiocyanate[d]	50 mg/kg	Rat 5M & 5F	20 days	Stomach: macroscopic—non-glandular part of the stomach was thickened, with lining occasionally roughened; microscopic—slight to moderate epithelial hyperplasia of the non-glandular part of the stomach, with acute to subacute ulcers from 2 to 6 1/2 mm across observed in all animals. Liver: microscopic—minor inflammatory foci
	20 mg/kg	do.	20 days	Stomach: macroscopic—same as seen in the 50 mg/kg animals; microscopic—the same type of ulceration as seen in the higher dose, occurring in 50% of the animals
Amyl butyrate	10,000	10M & 10F	16 wk	No effect
(pentyl butyrate)	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Amyl valerianate	10,000	do.	13 wk	No effect
(pentyl pentanoate)	1000	do.	do.	No macroscopic effect
Anethole	10,000	5M & 5F	15 wk	Liver: microscopic—slight hydropic changes of hepatic cells in males only
(1-methoxy-4-propenylbenzene)	2500	do.	1 yr	No effect
Anisaldehyde	10,000	do.	15 wk	No effect
(*p*-methoxybenzaldehyde)	1000	do.	27–28 wk	do.
Benzaldehyde	10,000	do.	16 wk	do.
	1000	do.	27–28 wk	do.
Benzyl cinnamate	10,000	do.	19 wk	do.
(cinnamein, benzyl β-phenylacrylate)	1000	do.	do.	No macroscopic effect
Calamus oil	10,000[e]	10M & 10F	18 wk	Dose-related growth depression, greater in males than in females. Increased mortality. Large amounts of clear fluid in the abdominal cavity. Liver: macroscopic—variable in appearance with some lobes reduced in size with rough surfaces and others smooth, abnormally rounded, and enlarged; microscopic—variation in cell size, capsular thickening, proliferation of bile duct epithelium, portal area fibrosis with haemosiderin deposition (females more severely affected than males). Heart—minimal to slight myocardial degeneration characterized by varying degrees of necrosis of muscle fibres, early fibrosis, and infiltration with mononuclear cells

[e]Young adult animals were used.

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Calamus oil	5000	Rat[e] 10M & 10F	18 wk	Growth depression. Moderate amounts of clear fluid in the abdominal cavity. Liver—same changes as with 10,000 ppm but less severe. Heart—same changes as with 10,000 ppm but lower incidence
do.	2500	do.	do.	Growth depression. Liver—same changes as with 5000 and 10,000 ppm but less severe. Heart—same changes seen with the 10,000 ppm dose were seen in 1 of 6 rats examined
	1000	5M & 5F	do.	Growth depression
Carvone	10,000	do.	16 wk	Growth retardation, testicular atrophy
(*p*-mentha-6,8-dien-2-one)	2500	do.	1 yr	No effect
	1000	do.	27–28 wk	do.
Cinnamic aldehyde (cinnamaldehyde)	10,000	10M & 10F	16 wk	Liver—slight hepatic cell swelling. Stomach—slight hyperkeratosis of squamous portion
	2500	do.	do.	No effect[f]
	1000	do.	do.	No macroscopic effect
Citral	10,000	do.	13 wk	No effect
(3,7-dimethyl-2,7-octadienal)	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Coumarin[g,h] (2*H*-1-benzopyron-2-one)	10,000[e]	3M & 3F	4 wk	Marked growth retardation, testicular atrophy. Liver: microscopic—slight to moderate damage consisting of dead and dying cells, decrease in oxyphilia and cytoplasm in the centrolobular cells, and proliferation of bile ducts
	10,000	10M & 10F	1–8 wk	Marked growth retardation. No animal survived beyond 8 wk. Liver: microscopic—slight to moderate damage of the type seen with the 10,000 ppm dose for 4 wk

[f]Microscopic examinations were made on the stomach only.
[g]Toxicity study will be presented in a separate report.
[h]Not acceptable for food use because of toxic effects observed in chronic feeding studies.

Table 1 continued

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Coumarin[g,h] (2*H*-1-benzopyron-2-one)	1000	Rat 10M & 10F	14 wk	No effect
	1000	5M & 5F	28 wk	do.
	2500	6M & 6F	29 wk	Growth retardation. Liver: macroscopic—slight mottling in the males; microscopic—slight mid-zonal fatty change in both sexes but more marked in the males
	5000[i]	do.	2 yr	Growth retardation. Normal food utilization (measured for first year). Haemogram-decrease in haemoglobin. Liver: macroscopic—enlargement, distortion by poorly to well delineated masses, pale pin-point foci; microscopic—focal proliferation of bile ducts of atypical appearance with associated fibrosis (cholangiofibrosis) situated in the masses described grossly and not clearly delineated from the surrounding hepatic parenchyma; slight degree of fatty metamorphosis, very slight to slight architectural irregularity, very slight to slight variation in cell size, very slight proliferation of bile ducts of the normal type, minimal focal necrosis
	2500[i]	5M & 7F	2 yr	Liver—slight damage of the same type as seen with the 5000 ppm dose; cholangiofibrosis was not seen
	2500	6M & 6F	do.	Liver—minimal damage of the same type as seen with 5000 ppm dose; cholangiofibrosis was not seen
	1000[i]	do.	do.	No effect

[i]Corn oil added to diet at a level of 3% (w/w). Two control groups were included in the study. Corn oil was added to the diet of one control group; the diet of the other group was untreated.

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Coumarin[a,b] (2*H*-1-benzopyron-2-one)	100 mg/kg	Dog 1M & 1F	9–16 days	Male killed in extremis after 9 days. Female found dead on day 16. Marked emaciation, slight dehydration, and slight jaundice. Liver: macroscopic—moderate to marked yellow colour and moderate "nutmeg" appearance; microscopic—marked disorganization of the lobular pattern, moderate increase in size of liver cells (with variation ranging from slight atrophy to a great increase), slight to moderate vacuolation, moderate to large amount of diffusely distributed fat, slight to moderate focal necrosis, fibrosis ranging from slight (centrolibular) to moderate to marked (portal) and very slight to moderate bile-duct proliferation. Spleen—macroscopic moderately pale. Gastro-intestinal tract—intestinal contents thick, dark, and tarry. Bone marrow—thin and fatty. Gall bladder—moderately distended
	50 mg/kg	2M & 1F	35–277 days	Emaciation; slight to moderate jaundice in the two female dogs. One death at 35 days; remaining dogs sacrificed at intervals between 45 and 277 days. Liver: macroscopic—moderately yellow colour with "nutmeg" appearance; microscopic—the same changes as with the 100 mg/kg dose except that the liver cell vacuolation was greater. Spleen—large amount of haemosiderin. Bone marrow—pale
	25 mg/kg	do.	133–330 days	Moderate emaciation and slight jaundice in one female. Weight gain in remaining dogs. Liver—slightly pale in two dogs, dark red in a third and yellow, markedly "nutmeg" in the fourth; microscopic—liver changes like those seen on the 50 mg/kg dose to the same degree in one dog and less severe in the other three. Spleen—moderate haemosiderosis. Gall bladder—moderately distended in two animals
	10 mg/kg	2M & 2F	297–350 days	No definite effect
Dihydroanethole (1-methoxy-4-propylbenzene)	10,000	Rat 10M & 10F	19 wk	Bone—slight osteoporosis of the tibia and femur (6/10)
	2500	do.	do.	Bone—slight to very slight osteoporosis (6/10)
	1000	do.	do.	Bone—very slight osteoporosis[j] (4/10)

[j]In the control group slight osteoporosis was seen in one of 10 animals.

Table 1 continued

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Dihydroanethole (1-methoxy-4-propylbenzene)	Maximal dose 5000 mg/kg	Rat 20M	32 days	Initial dose 2000 mg/kg increased gradually to 5000 mg/kg. Seven of the animals survived the full 32 days of treatment and 16 lived long enough to receive the maximal daily dose of 5000 mg/kg Stomach: macroscopic—mucosa of the forestomach showed coalescent areas covered by thick flaky white material punctuated by minute ulcers; microscopic—moderately severe degree of hyperkeratosis of the stratified squamous epithelium in the forestomach. Bone—moderate degree of osteoporosis
Dihydrocoumarin (1,2-benzohydropyrone)	10,000	10M & 10F	14 wk	No effect
	1000	do.	do.	do.
	150 mg/kg	Dog 1F — ?	2 yr	do.
	50 mg/kg	1M & 1F	do.	do.
Dihydrosafrole[b] (1,2-methylenedioxy-4-propylbenzene) (Long & Jenner, 1963) (Hagan *et al.* 1965)	10,000	Rat 10M & 10F	do.	Growth retardation in both sexes. Oesophagus—benign epidermoid papillomas and malignant papillary epidermoid carcinomas; 75% of the rats showed oesophageal tumours, 50% of the tumours were malignant. Liver: macroscopic—enlargement; microscopic—slight damage of the same type as that seen in the safrole-treated rats (see safrole rat—2-yr feeding study). Spleen—moderate follicular atrophy
	5000	25M & 25F	do.	Growth retardation in both sexes. Oesophagus—74% of the rats showed oesophageal tumours, 32% of the tumours were malignant. Liver: macroscopic—enlargement; microscopic—slight damage of the same type as seen in the safrole-treated rats. Spleen—moderate follicular atrophy
	2500	10M & 10F	do.	Growth retardation in the females. Oesophagus—20% of the rats showed oesophageal tumours, 5% of which were malignant. Liver: macroscopic—enlargement; microscopic—slight liver damage of the same type as seen in the safrole-treated rats. Kidney—moderate increase in chronic nephritis

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Dihydrosafrole[h] (1,2-methylenedioxy 4-propylbenzene) (Long & Jenner, 1963) (Hagan *et al.*1965)	1000	Rat 10M & 10F	2 yr.	Growth retardation in the females. Liver: macroscopic—slight enlargement; microscopic—slight liver damage of the same type as that seen in the safrole-treated rats. Kidney—moderate increase in chronic nephritis
Dimethylbenzyl carbinol (1,1-dimethyl-2-phenylethanol)	10,000	5M & 5F	16 wk	No effect
	1000	10M & 10F	28 wk	do.
Dolcourin[k]	10,000	5M & 5F	16 wk	do.
Ethyl caprylate (ethyl penanoate)	10,000	10M & 10F	17 wk	do.
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Ethyl formate	10,000	do.	17 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Ethyl methylphenyl glycidate (ethyl ester of 2,3-epoxy-3-methyl-3-phenylpropionic acid)	10,000	5M & 5F	16 wk	Growth retardation most obvious in males Testes—marked atrophy
	2500	do.	1 yr	No effect
Ethyl oenanthate (ethyl heptanoate)	10,000	10M & 10F	13 wk	do.
	1000	do.	do.	No macroscopic effect
Ethyl oxyhydrate[l]	10,000	do.	17–18 wk	Liver: microscopic—very slight swelling of hepatic cells in males
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Ethyl pelargonate (ethyl nonanoate)	10,000	5M & 5F	16 wk	No effect
Ethyl sebacate	10,000	do.	17–18 wk	do.
	1000	do.	27–29 wk	do.
Ethyl vanillin (3-ethoxy-4-hydroxybenzaldehyde)	50,000[m]	5M	1 yr	do.
	20,000[m]	do.	do.	do.
	20,000[n]	12M & 12F	2 yr	do.
	10,000[n]	do.	do.	do.
	5000[n]	do.	do.	do.
Eugenol (1-hydroxy-2-methoxy-4-allylbenzene)	10,000	10M & 10F	19 wk	do.
	1000	do.	do.	No macroscopic effect

[k]Dolcourin is a trade mark name for a substance supplied by Dodge and Olcott, Inc. as a coumarin substitute.
[l]Ethyl oxyhydrate is a mixture whose composition varies with the method of manufacture. The material used in this study was obtained from Florasynth Labs., Inc., N.Y.
[m]3% (w/w) corn oil added to control and test diets as a binder to reduce evaporation of the flavouring.
[n]3% (w/w) propylene glycol added to control and test diets as a binder to reduce evaporation of the flavourings.

Table 1 continued

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Eugenol (1-hydroxy-2-methoxy-4-allylbenzene)	Maximal dose 4000 mg/kg	Rat 20M	34 days	Initial dose 1400 mg/kg increased gradually to 4000 mg/kg. Eight of the animals survived the full 34 days of treatment and 15 lived long enough to receive the maximal dose of 4000 mg/kg. Stomach: macroscopic—mucosa of the forestomach showed coalescent areas covered by thick flaky white material punctuated by minute ulcers; microscopic—moderately severe degree of hyperkeratosis of the stratified squamous epithelium in the forestomach. Bone—small degree of osteoporosis
Eugenyl acetate (1-acetoxy-2-methoxy-4-allylbenzene)	10,000	10M & 10F	19 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Geraniol extra (3,7-dimethyl-2,6-octadienol) (3,7-dimethyl-1,6-octadienol)	10,000	5M & 5F	16 wk	No effect
	1000	do.	27–28 wk	do.
Geranyl acetate	10,000	10M & 10F	17 wk	do.
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Ionone standard [60% α-ionone, 4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one and 40% β-ionone, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one]	10,000	do.	do.	Liver: microscopic—slight to moderate swelling of parenchymal cells
	2500	do.	do.	Liver: microscopic—slight amount of swelling of parenchymal cells[e]
	1000	do.	do.	Liver: microscopic—very slight amount of swelling of the parenchymal cells[e]
Isoeugenol (1-hydroxy-2-methoxy-4-propenylbenzene)	10,000	5M & 5F	16 wk	No effect
Isosafrole[h]	10,000	10M & 10F	11 wk	Growth retardation in both sexes. No rats on this dose survived beyond 11 wk of treatment. Liver: macroscopic—enlargement; microscopic—slight damage of the same type as seen with safrole
(Hagan *et al.* 1965)	5000	25M & 25F	2 yr	Growth retardation in both sexes. Testes—increased number of interstitial cell tumours. Liver: macroscopic—enlargement; microscopic—slight damage of the same type as seen with safrole. Five rats had primary hepatic tumours (two of these had hepatic cell adenomas and three had hepatic cell carcinomas). Kidney—increased incidence of chronic nephritis. Thyroid—slight hyperplasia

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Isosafrole[b] (Hagan *et al.* 1965)	2500	Rat 10M & 10F	2 yr	Slight growth retardation in females. Liver: macroscopic—slightly enlarged; microscopic—slight damage of the same type as seen with safrole, no primary hepatic tumours. Thyroid—slight hyperplasia
	1000	do.	do.	Slight growth retardation in females. Liver: microscopic—slight damage of the same type as seen with safrole
Linalyl cinnamate	10,000	10M & 10F	17 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Linalyl isobutyrate	10,000	do.	18 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Methyl anthranilate (*o*-aminobenzoic acid, methyl ester)	10,000	do.	13 wk	No effect
	1000	do.	do.	No macroscopic effect
6-Methylcoumarin (6-methyl-2*H*-1-benzopyran-2-one)	15,000	25M & 25F	2 yr	Depression of growth, severe in males and moderate in females, and paralleled by decrease in food intake. Liver: microscopic—slight fatty metamorphosis, and very slight bile-duct proliferation and focal telangiectasia. Testes—moderate atrophy, probably due to growth retardation
	7500	do.	do.	Depression of growth in males
	3500	do.	do.	No effect
	1000	do.	do.	do.
	500	do.	do.	do.
	10,000	10M & 10F	14 wk	do.
	1000	do.	do.	do.
	150 mg/kg	Dog 1M	39 days	Sacrificed because of weakness, emaciation, dehydration. Liver: microscopic—moderate to severe hepatitis. Skeletal muscle—slight to moderate atrophy
	50 mg/kg	1M & 1F	2 yr	No effect

Table 1 continued

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Methyl salicylate (*o*-hydroxybenzoic acid, methyl ester)	20,000	Rat 24M & 26F	49 wk	Growth retardation, rough coats. All dead by wk 49. Bone: microscopic—excess of cancellous tissue
(Webb & Hansen, 1963)	10,000	25M & 25F	2 yr	Growth retardation, rough coats. Bone: microscopic—slight excess of cancellous tissue
	5000	do.	do.	Bone: microscopic—slight excess of cancellous tissue
	1000	do.	do.	No effect
	10,000	10M & 10F	17 wk	Growth retardation
	1000	do.	do.	No macroscopic effect
	1200 mg/kg	Dog 1M & 1F	3 days	Died, weight loss. Liver: macroscopic—slightly pale, "nutmeg" in appearance; microscopic—moderate to marked amount of fat
	800 mg/kg	1M	29 days	Sacrificed, weight loss. Liver: microscopic—trace of fat
	800 mg/kg	1F	4 days	Died, weight loss. Liver: macroscopic—slightly pale, "nutmeg" in appearance; microscopic—moderate to marked amount of fat
	500 mg/kg	1M & 1F	8–9 days	Sacrificed—No effect
	250 mg/kg	do.	51–52 days	do.
	100 mg/kg	do.	52–53 days	do.
	50 mg/kg	do.	53–59 days	do.
	350 mg/kg	2M & 1F	2 yr	Weight loss. Liver: macroscopic—enlarged; microscopic—enlarged hepatic cells
	150 mg/kg	2M & 2F	do.	Growth retardation. Liver: macroscopic—enlarged; microscopic—enlarged hepatic cells
	50 mg/kg	do.	do.	No effect
Phenylethyl phenylacetate	10,000	Rat 10M & 10F	17 wk	do.
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Piperonal (3,4-methylenedioxybenzaldehyde)	10,000	5M & 5F	15 wk	No effect
	1000	do.	27–28 wk	do.

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Safrole[b] (1,2-methylenedioxy-4-allylbenzene) (Hagan *et al.* 1965)	10,000	Rat 10M & 10F	62 wk	Growth retardation in both sexes. All rats dead by wk 62. Testes—atrophy. Stomach—atrophy and atypical regeneration of the mucosa glands with associated fibrosis and hyalinization of the surrounding stroma most severe at the base. Liver: macroscopic—enlarged (2 to 3 times the normal size), mottled and irregularly nodular, with single or multiple tumour masses; microscopic—hepatic cell enlargement, which was usually focal and resulted in the formation of nodules. The enlargement was chiefly of the cytoplasm, which appeared normal in some cells but showed fatty metamorphosis or peripheral migration of the basophilic cytoplasmic granules in other cells. The nodules tended to progress in one of 3 ways (sometimes all 3 were seen in the same liver): (1) cystic necrosis; (2) cirrhosis; and (3) adenomatoid hyperplasia, leading to the formation of both benign and malignant tumours (hepatic cell adenoma, hepatic cell carcinoma, hepatocholangioma, hepatocholangiocarcinoma)
	5000	25M & 25F	2 yr	Growth retardation in both sexes. Increased mortality in the males. Liver—changes of the same type and severity as seen with the 10,000 ppm dose except for the occurrence of macroscopic cysts and a higher incidence of tumours in the lower dose, statistically-significant increase in malignant primary hepatic tumours. Stomach—same type of changes as with 10,000 ppm dose. Kidney—slight increase of chronic nephritis in the females. Thyroid—mild hyperplasia
	2500	10M & 10F	do.	Growth retardation in both sexes. Liver—moderate liver damage of the same type as seen with the 10,000 ppm dose with the exception of cirrhosis. Kidney—moderate increase of incidence of chronic nephritis.

Table 1 continued

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Safrole[b] (1,2-methylenedioxy-4-allylbenzene) (Hagan *et al.* 1965)	1000	Rat 10M & 10F	2 yr	Growth retardation in the females. Liver—slight liver damage of the type seen with the 10,000 ppm dose, except that malignant tumours and cirrhosis were not seen with this dose. Kidney—moderate increase in chronic nephritis in the males.
(Long *et al.* 1963)	5000	25M & 25F	do.	Growth retardation in both sexes. Haemograms—mild anaemia, slight leucocytosis. Liver—marked liver changes (including malignant tumours but only rare cirrhosis) of the type seen with the 10,000 ppm dose. Statistically-significant increase in benign and malignant primary liver tumours.
	1000	25M & 25F	do.	Liver—slight to moderate damage of the same type seen with the 10,000 ppm dose except that malignant tumours and cirrhosis were not seen
	500	do.	do.	Liver—slight damage of the same type seen with the 10,000 ppm dose except that cirrhosis was not seen
	100	do.	do.	Liver—very slight damage of the same type seen with the 10,000 ppm dose except that cirrhosis was not seen
	80 mg/kg	Dog 2M & 2F	26–39 days	One dog died; the remaining dogs were sacrificed in a moribund condition. Marked weight loss. Skeletal muscle—moderate to severe atrophy in 2 of 4 dogs. Liver: macroscopic—slight enlargement in 2 of 4 dogs; microscopic—heavy infiltration of fat, occasional bile-duct proliferation and inflammatory-cell infiltration, slight to moderate streaking with necrobiosis and liver cell atrophy
	40 mg/kg	do.	91–116 days	Emaciation, general weakness. Liver: macroscopic—slight enlargement in 3 of 4 dogs; microscopic—slight fat infiltration, moderate hepatic cell atrophy and necrobiosis as at 80 mg/kg

Compound	Dose level (ppm)[a]	Species, group size and sex	Duration of test	Effects
Safrole[b] (1,2-methylenedioxy-4-allylbenzene (Long *et al.* 1963)	20 mg/kg	Dog 2M & 2F	6 yr	Liver: macroscopic—slight to moderate enlargement, nodular surface; microscopic—mild post-necrotic cirrhosis characterized by focal or generalized nodules, consisting of enlarged hepatic cells with slight focal fatty vacuolization, separated by bands of atrophied hepatic cells and collagen; slight lymphocytic, Kupffer cell and bile-duct proliferation; minimal focal necrosis. Thyroid—hyperplasia
	5 mg/kg	do.	do.	Liver: microscopic—minimal focal necrosis, bile-duct proliferation, fatty metamorphosis, hepatic cell atrophy and leucocytic infiltration
Terpinyl acetate (*p*-menth-1-en-8-ol ester of acetic acid)	10,000	Rat 10M & 10F	20 wk	No effect
	2500	do.	do.	No macroscopic effect
	1000	do.	do.	do.
Thymol (*p*-cymen-3-ol; hydroxy-*p*-cymene)	10,000	5M & 5F	19 wk	No effect
	1000	do.	do.	No macroscopic effect
γ-Valerolactone (4-hydroxypentanoic acid lactone)	10,000	10M & 10F	13 wk	No effect
	5000	do.	do.	do.
	2500	11M & 9F	do.	do.
	1000	10M & 10F	do.	do.
Vanillin (4-hydroxy-3-methoxybenzaldehyde)	50,000[m]	5M	1 yr	do.
	20,000[m]	do.	do.	do.
	20,000[n]	12M & 12F	2 yr	do.
	10,000[n]	do.	do.	do.
	5000[n]	do.	do.	do.
	10,000	5M & 5F	16 wk	do.
	1000	do.	27–28 wk	do.

Table 2. *Loss of food flavourings from laboratory animal diets during a 7-day period*

Flavouring	Percentage loss*	Flavouring	Percentage loss*
Acetophenone	31	Eugenyl acetate†	6
Allylcaproate	31	Geranyl acetate†	4
Amyl butyrate†	35	Ionone†	1
Anethole	6	Isosafrole	5
Calamus oil	0	Linalyl cinnamate	4
Cinnamic aldehyde	14	Linalyl isobutyrate	7
Citral	58	Methyl salicylate†	3 & 5§
Dihydroanethole†	16	Phenylethyl phenylacetate	0
Dihydrocoumarin	—‡	Safrole	12
Dihydrosafrole	6	Terpinyl acetate	7
Ethyl caprylate†	33		

*100−(100 × day 7 recovery/day 0 recovery).
†Reported by Jones *et al.* (1962). Recovery data on the remaining compounds were supplied by W. I. Jones.
‡Dihydrocoumarin was partially converted to melilotic acid when mixed with the laboratory diet; the test animals ingested a mixture of the two compounds.
§The two values for percentage loss indicate two different analytical procedures.

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Substances d'assaisonnement et composés de structure connexe. II. Toxicité subaiguë et chronique

Résumé—On a étudié sur des rats la toxicité subaiguë et chronique de 48 substances d'assaisonnement. On a également étudié sur des chiens la toxicité de cinq d'entre elles (coumarine, 6-méthylcoumarine, dihydrocoumarine, salicylate de méthyle et safrol). On a présenté un résumé de ces études. On a déterminé aussi la depérdition de 21 substances d'assaisonnement en 7 jours, dans des régimes pour animaux de laboratoire.

Lebensmittel-Geschmacksstoffe und Verbindungen verwandter Struktur. II. Subakute und chronische Toxizität

Zusammenfassung—Die subakute und bzw. oder chronische Toxizität von 48 Lebensmittel-Geschmackszusätzen wurde an Ratten untersucht. Die Toxizität von fünf dieser Geschmacksstoffe (Cumarin, 6-Methylcumarin, Dihydrocumarin, Methylsalicylat und Safrol) wurde auch an Hunden untersucht. Eine Zusammenfassung der Versuchsergebnisse wird vorgelegt. Der Verlust von 21 Geschmacksstoffen aus dem Futter von Laboratoriumstieren während einer 7-Tage-Periode wurde ebenfalls bestimmt.

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Dietary Fats and Plasma Lipids in Chicks.* (28633)

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In studies with growing chicks fed cholesterol and fats, Adamson *et al.*(1) observed that unsaturated fat caused greater increase in serum and liver cholesterol than saturated fat. We have previously reported that u saturated fats like sesame oil and mustar

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TABLE I. Plasma Lipids of Chicks Fed Different Diets for 8 Weeks.

Treatment		Total cholesterol (mg %)	Beta lipoprotein cholesterol (mg %)	Phospho-lipid (mg %)	Triglyc-erides (mg %)	Non-ester fatty acids (μEq/l)	Lipoprotein Alpha (%)	Lipoprotein Beta (%)
Basal diet	(6)	141 ± 12	76 ± 5	246 ± 17	81 ± 9	252 ± 34	63 ± 3	37 ± 3
Basal + cholesterol	(6)	259 ± 29	132 ± 14	255 ± 27	94 ± 10	338 ± 72	48 ± 4	52 ± 5
Basal + mustard oil	(5)	158 ± 7	50 ± 6	274 ± 7	103 ± 15	534 ± 57	72 ± 4	28 ± 4
Basal + cholesterol	(5)	400 ± 59	240 ± 57	264 ± 13	119 ± 4	926 ± 50	30 ± 4	70 ± 4
Basal + coconut oil	(6)	174 ± 10	65 ± 6	322 ± 15	89 ± 20	445 ± 47	69 ± 2	31 ± 2
Basal + cholesterol	(6)	634 ± 83	335 ± 74	279 ± 27	158 ± 25	679 ± 74	26 ± 3	74 ± 3
Basal + sesame oil	(5)	164 ± 20	77 ± 3	300 ± 34	93 ± 13	347 ± 20	66 ± 2	34 ± 2
Basal + cholesterol	(6)	735 ± 82	497 ± 35	295 ± 10	161 ± 15	552 ± 41	21 ± 2	79 ± 2
Basal + hydrogenated groundnut oil	(6)	158 ± 11	50 ± 4	276 ± 20	70 ± 4	510 ± 43	70 ± 2	30 ± 3
Basal + cholesterol	(5)	1036 ±127	741 ±113	340 ± 36	164 ± 17	585 ± 24	15 ± 3	85 ± 5

Figures in parentheses indicate No. of chicks used. Values = Mean ± S.E.

oil produced different effects on plasma cholesterol of monkeys(2,3). Sesame oil was found to be more hypercholesteremic than mustard oil. When rabbits were fed cholesterol dissolved in oil an inverse relation was reported between the increase in plasma cholesterol and the iodine number of the oils used as solvents(4). It has been suggested that absorption of dietary cholesterol might depend on its rate of solubility in dietary or endogenous fat(5).

This report concerns studies on the effects of feeding sesame oil, mustard oil, coconut oil and hydrogenated ground nut oil on plasma lipids of chicks. The possible relation between plasma cholesterol and solubility of cholesterol in the different oils was also studied.

Materials and methods. Male white Leghorn chicks were fed a basal diet(2) for 3 weeks and subsequently divided into various groups, as indicated in Table I. Oils when fed were mixed with the diet at 10% level. Cholesterol when administered was mixed with the diet at 1% level. Chicks were maintained on the different experimental diets for 8 weeks when blood was withdrawn by cardiac puncture using a heparinized syringe. Total cholesterol, β-lipoprotein cholesterol, lipoprotein percentage, phospholipid, triglycerides and nonesterified fatty acids (NEFA) of plasma were estimated(6).

To study the solubility of cholesterol in different oils, crystalline cholesterol was added to different oils taken in centrifuge tubes, stirred with glass rod, kept at 37°C in a constant temperature bath for 2 hours, centrifuged and aliquots of centrifugate used for estimation of cholesterol using the same method as that of plasma cholesterol.

Results. Addition of cholesterol in the basal diet without the oils increased the plasma levels of total cholesterol, β-lipoprotein cholesterol, and β-lipoprotein percentage. Addition of mustard oil, sesame oil and hydrogenated groundnut oil in the diet without addition of cholesterol increased the plasma NEFA values without significant alterations of the other plasma lipids. When coconut oil was added to the diet, increase in total cholesterol, NEFA and phospholipids of plasma was observed. Addition of cholesterol in the diet along with the different oils increased the plasma contents of total cholesterol, β-lipoprotein cholesterol, triglycerides, NEFA and β-lipoprotein percentage with a concomitant decrease in α-lipoprotein percentage. Maximum rise in total plasma cholesterol was observed in chicks fed cholesterol along with hydrogenated groundnut oil and sesame oil.

TABLE II. Solubility of Cholesterol in Different Oils at 37°C.

The oil	Iodine value of the oil	mg cholesterol dissolved/ml of the oil
Mustard oil	104	35.12*
Coconut oil	9	43.49
Sesame oil	110	40.83
Hydrogenated groundnut oil	55	38.96

* Avg of 3 observations.

The rise was minimum when mustard oil was fed along with cholesterol, with intermediate value when coconut oil and cholesterol were administered (Table I).

The solubility of cholesterol was highest with coconut oil and least with mustard oil (Table II).

Discussion. In experiments with chicks we observed a rise in plasma cholesterol after administration of the different oils along with cholesterol. The rise was maximum with sesame oil and minimum with mustard oil. Similar observations were recorded in our studies with rhesus monkeys. We, therefore, could not confirm the observation of Adamson *et al.*(1) as to the effect of unsaturated fat on serum cholesterol of chicks. Although there was not much difference in the solubilities of cholesterol in the different oils, the solubility was comparatively minimum in case of mustard oil. Comparatively lower rise in plasma cholesterol might be due to diminished absorption of cholesterol with mustard oil. This is corroborated by our previous observation on the increased excretion of fecal sterols after monkeys were fed cholesterol and mustard oil(2). The concentration of oleic acid in sesame oil and mustard oil is 49.4% and 32.3% respectively. It has been stated that oleic acid helps in absorption of cholesterol in rats and chicks(7-9). The lower rise in plasma cholesterol after mustard oil might, therefore, be due to its lower oleic acid content. Unlike monkeys, addition of cholesterol to the basal diet of chicks without any oils produced an increase in plasma cholesterol indicating absorption of cholesterol in the absence of oil. This indicated that the mechanism of absorption of cholesterol differs in the different species of animals. The increased plasma cholesterol in chicks fed only coconut oil might be due to the effect of the oil helping reabsorption of biliary or endogenous cholesterol as suggested by Wilkens *et al.*(5). Increased plasma NEFA values after administration of the different oils without cholesterol indicated that after absorption a fraction of fatty acids is transported in unesterified condition. When cholesterol was fed in addition to the different oils, there was a further increase in NEFA values of plasma. This might be due to altered carbohydrate metabolism as indicated previously(10).

Summary. Chicks were fed mustard oil, sesame oil, coconut oil and hydrogenated groundnut oil mixed with the basal diet with or without the addition of cholesterol. The level of oil was 10% and that of cholesterol 1% of the basal diet. The different diets were fed for 8 weeks and plasma contents of different lipids were estimated. Addition of oils alone in the diet increased the plasma NEFA values without producing any change in other fractions of plasma lipids. When the different oils were fed in addition to cholesterol, there was a rise in total plasma cholesterol, β-lipoprotein cholesterol, β-lipoprotein percentage, triglycerides and NEFA values of plasma. The rise in plasma cholesterol was maximum in chicks fed cholesterol along with hydrogenated oil or sesame oil and minimum in chicks fed cholesterol along with mustard oil, with intermediate rise after coconut oil. The solubility of cholesterol was highest in coconut oil and least in mustard oil. Hypercholesteremia does not seem to depend on saturation or unsaturation of the oils or on the solubility of cholesterol in the oils.

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Biochemical and Histochemical Changes in Aorta of Chicks Fed Vegetable Oils and Cholesterol. (30382)

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We reported earlier(1) that feeding vegetable oils and cholesterol to chicks for 8 weeks increased total plasma cholesterol, β-lipoprotein cholesterol, β-lipoprotein percentage, triglycerides, and free fatty acids in plasma. The rise in plasma cholesterol was maximum in chicks fed sesame oil with cholesterol or hydrogenated groundnut oil with cholesterol, minimum in chicks fed mustard oil with cholesterol with intermediate value when fed coconut oil with cholesterol. Hypercholesteremia did not depend on saturation or unsaturation of the oils. Clinical and experimental evidences indicate a direct relationship of atherosclerosis to the altered lipid metabolism characterized by hypercholesteremia and hyper β-lipoproteinemia. The origin of lipid in atheromatous plaques might be due to filtration from circulating plasma (2) or arterial synthesis of cholesterol(3-5) and phospholipids(6). Significant quantities of mucopolysaccharides containing hexosamine present in the arteries(7) are increased in rabbits fed cholesterol(8). Earliest change observed in the development of atherosclerosis was the marked accumulation of acid mucopolysaccharides(9).

We wished to investigate the interrelation, if any, between dietary fat, plasma lipids and changes in the various parts of the aorta. The distribution of elastic tissue, acid mucopolysaccharides and lipids was, therefore, determined histochemically and cholesterol, phospholipids, hexosamine and hydroxyproline were determined chemically in the different segments of the aorta in chicks fed vegetable oils and cholesterol.

Materials and methods. Two-week-old male white Leghorn chicks were fed a basal diet(10) for 3 weeks, then divided into groups of 6 birds (Table I). Oils when fed were mixed with the diet at 10% level. Cholesterol when administered was mixed with the diet at 1% level. Average daily food consumption by each chick was 30 g. After the diets were fed for 8 weeks, birds were killed

TABLE I. Plasma Cholesterol and Arterial Cholesterol, Phospholipids, Hexosamine and Hydroxypro[illegible] Chicks Fed Different Diets for 8 weeks.

Treatments	Total plasma cholesterol, mg %	Arterial cholesterol* (mg/100 g wet wt) Arch	Arterial cholesterol* (mg/100 g wet wt) Tho + abd	Arterial phospholipid* (mg/100 g wet wt) Arch	Arterial phospholipid* (mg/100 g wet wt) Tho + abd	Arterial hexosamine† (mg/100 g dry wt) Arch	Arterial hexosamine† (mg/100 g dry wt) Tho + abd	Arterial hydroxyp[illegible] (mg/100 g dry wt) Arch	[illegible]
Basal diet	141 ±12	70	97	149	130	1.62 ±.05	1.14 ±.04	1.33 ±.09	[illegible]
Idem + cholesterol	259 ±29	97	132	178	148	1.60 ±.06	1.28 ±.03	1.55 ±.07	[illegible]
Basal + mustard oil	158 ± 7	68	48	191	192	1.26 ±.11	1.07 ±.04	1.41 ±.06	[illegible]
Idem + cholesterol	400 ±59	190	240	176	205	1.30 ±.05	1.09 ±.08	1.50 ±.06	[illegible]
Basal + coconut oil	174 ±10	71	60	223	213	1.25 ±.04	1.12 ±.03	1.29 ±.02	[illegible]
Idem + cholesterol	634 ±83	158	207	137	255	1.39 ±.03	1.04 ±.03	1.51 ±.14	[illegible]
Basal + sesame oil	164 ±20	75	92	196	161	1.54 ±.04	1.19 ±.03	1.37 ±.02	[illegible]
Idem + cholesterol	735 ±82	198	299	293	364	1.58 ±.12	1.14 ±.13	1.55 ±.06	[illegible]
Basal + hydrogenated groundnut oil	158 ±11	64	72	173	175	1.34 ±.10	1.11 ±.07	1.59 ±.14	[illegible]
Idem + cholesterol	1036 ±127	173	251	140	271	1.15 ±.05	1.01 ±.02	1.65 ±.17	[illegible]

* Mean of 2 determinations from pooled aorta. † Values are Mean ± S.E. (5 chicks in each group

by decapitation after withdrawal of blood by cardiac puncture. The aorta was removed, separated into arch and thoracico-abdominal portions, freed from adherent blood and connective tissue and cut into various pieces. A few pieces were used for estimation of hexosamine(11) and hydroxyproline(12). The aortic segments of 2 or more birds were pooled, dried, extracted in a hot extractor with pretroleum ether for 12 hours and the extract used for estimation of cholesterol and phospholipids(13). Plasma cholesterol was also estimated(13). The aorta of one chick in each group was used for histochemical studies. Arterial segments were fixed in 10% neutral formol. Frozen sections were stained with Sudan Black B for lipids(14) and paraffin sections were stained for elastic tissue(15) and acid mucopolysaccharides(16).

Results. Cholesterol: In both the control and experimental chicks the arch of aorta contained more cholesterol than the thoracico-abdominal portion. Minimum changes were produced when chicks were fed cholesterol or oil. Aortic cholesterol increased 2 to 3 [illegible] when cholesterol was fed along with [illegible] *Phospholipids:* The distribution was un[illegible] in the different segments of aorta. Fe[illegible] oils with or without cholesterol, inc[illegible] phospholipids in most of the chicks. M[illegible] mum increase was observed in chick[illegible] sesame oil and cholesterol, the thora[illegible] abdominal portion containing a higher a[illegible] than the arch of the aorta. *Hexosamine:* both control and experimental chick[illegible] osamine was higher in the arch of the [illegible] than in the thoracico-abdominal aorta. H[illegible] osamine decreased in the aortic arch [illegible] chicks were fed coconut oil, mustard o[illegible] hydrogenated groundnut oil with or w[illegible] cholesterol and in the thoracico-abd[illegible] aorta when cholesterol was fed with c[illegible] oil or hydrogenated groundnut oil. [illegible] hexosamine did not change when sesa[illegible] was fed with or without cholesterol. *H[illegible] proline:* In control and most of the [illegible] mental groups hydroxyproline of aort[illegible] was less in the arch of aorta than in

TABLE II. Distribution of Acid Mucopolysaccharides and Lipids in Aorta of Chicks Fed Different Diets for 8 Weeks.

Treatments	Acid mucopolysaccharides Arch	Thor	Abd	Lipids Arch	Thor	Abd
Basal diet	+	+	+	—	—	—
Idem + cholesterol	+	+	+	—	—	—
Basal + mustard oil	+	++++	+	—	—	—
Idem + cholesterol	++	++++	+	—	+	—
Basal + coconut oil	++	++	+	—	—	—
Idem + cholesterol	+	+	+	++	++	—
Basal + sesame oil	+	+	+	—	—	—
Idem + cholesterol	+++	+	+	+	+	++
Basal + hydrogenated groundnut oil	+	+	++++	—	—	—
Idem + cholesterol	+	++++	+	+++	++++	++

thoracico-abdominal aorta. Hydroxyproline increased in the aortic arch when cholesterol was fed with sesame oil, and in the thoracico-abdominal portion when cholesterol was fed with coconut oil, sesame oil or hydrogenated groundnut oil. Mustard oil feeding did not alter arterial hydroxyproline, neither did it change when oils or cholesterol were fed alone. *Elastic tissue:* Feeding of cholesterol or oils did not affect the elastic tissue of the aorta. Slight proliferation of intima with little disruption of elastic tissue was seen in the arch and thoracic aorta when cholesterol was fed along with coconut oil, mustard oil and hydrogenated groundnut oil. Feeding of cholesterol with sesame oil produced atheromatous plaque, fragmentation of elastic tissue throughout intima and complete disappearance from the media of the arch of aorta with slight intimal proliferation and fraying of the intimal elastic tissue in the thoracic aorta. No change in the abdominal aorta was seen after the chicks were fed different diets. *Lipid:* Lipid was either absent or present in a very low concentration in the aorta which could be detected by histochemical methods. Feeding vegetable oils or cholesterol did not produce any change. Lipid was deposited when cholesterol was fed with oils in the intima. The deposition was marked in the arch and thoracic aorta when coconut oil was fed, in abdominal aorta when sesame oil was fed and in thoracic aorta when mustard oil or hydrogenated groundnut oil was fed. *Acid mucopolysaccharides:* Feeding of cholesterol alone did not produce any change in its distribution. Coconut oil feeding increased it in intima and media of arch and thoracic aorta and decreased it in abdominal aorta. Sesame oil fed with cholesterol increased acid mucopolysaccharide in the aortic arch. Mustard oil fed alone or with cholesterol increased acid mucopolysaccharide only in the aortic arch. Feeding hydrogenated groundnut oil increased it in abdominal aorta and in the thoracic aorta when cholesterol was fed simultaneously. Detailed results are given in Tables I and II and in Fig. 1-21.

Discussion. Arterial cholesterol and phospholipids: Increased aortic cholesterol and phospholipids in chicks fed cholesterol with or without vegetable oils confirmed earlier reports(17). Elevation of arterial lipids might be due to increased lipogenesis(18), increased transfer of plasma lipids(19) or decreased destruction as a result of decreased lipolytic activity consequent to deficient production of heparin(20).

Arterial hexosamine and hydroxyproline: Hexosamine value represented the conibined hexosamine content of mucoproteins, mucoids and glycoproteins of the aorta. Diminution of arterial hexosamine after feeding the oils with or without cholesterol indicated that changes were not related with the hypercholesteremia. Mucopolysaccharides act as precursors in formation of collagen fibrils(21). Intimal and subintimal fibrosis characteristic of atheromatous lesions has been suggested to be due to depolymerization of mucopolysaccharides(22) and as a result mucopolysaccharides are decreased. We have also obtained decreased hexosamine in the aorta. Decreased mucopolysaccharide in aorta will

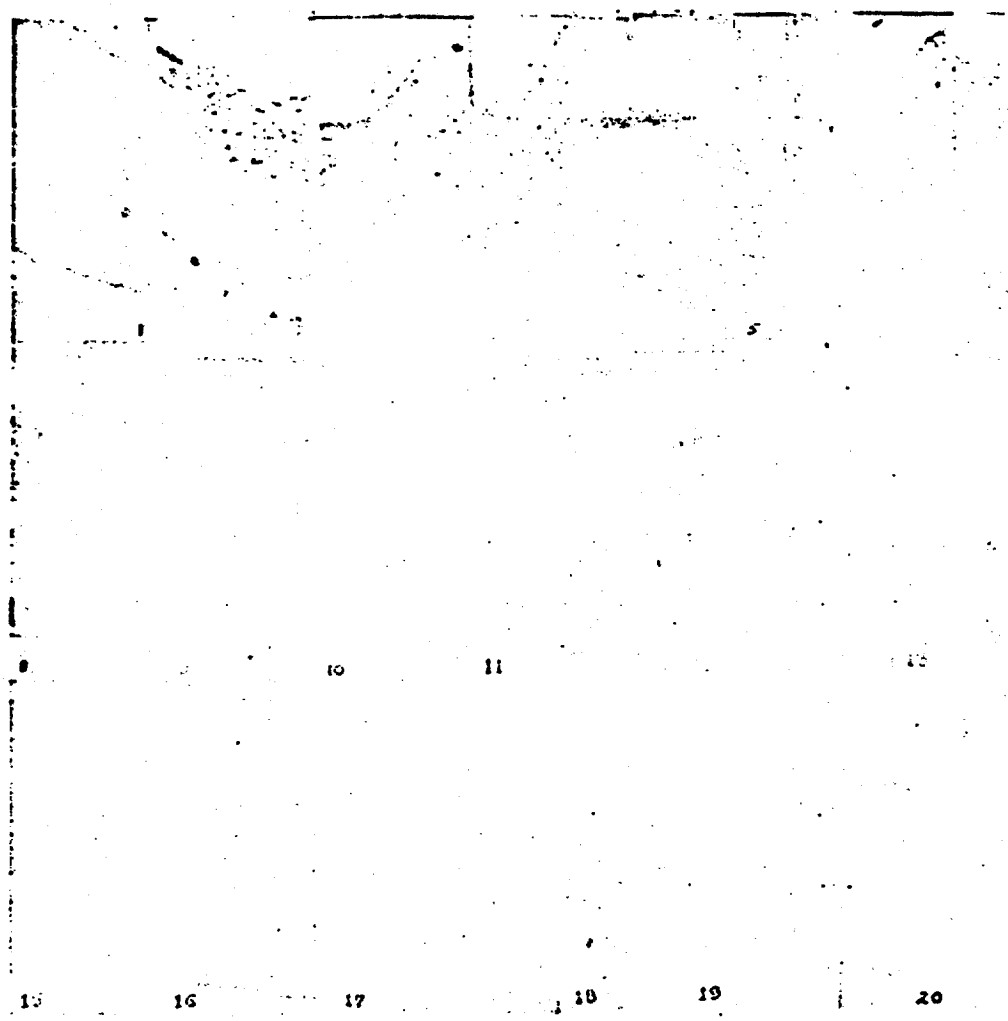

FIG. 1-7 showing distribution of elastic tissue in aorta. × 56.

Fig. 1. Arch of aorta in a chick fed basal diet.

Fig. 2. Arch of aorta in a chick fed coconut oil + cholesterol showing slight intimal proliferation and disruption of elastic tissue.

Fig. 3. Arch of aorta in a chick fed sesame oil + cholesterol showing atheromatous plaque, fragmentation of the elastic tissue in the intima.

Fig. 4. Arch of aorta in a chick fed mustard oil + cholesterol showing similar changes as in Fig. 2.

FIG. 5. Arch of aorta in a chick fed hydrogenated groundnut oil and cholesterol showing similar changes as in Fig. 2.

FIG. 6. Thoracic aorta in a chick fed coconut oil and cholesterol showing little fragmentation of the elastic tissue in intima and proliferation of the intima.

FIG. 7. Thoracic aorta in a chick fed sesame oil and cholesterol showing intimal proliferation and fraying of intimal elastic tissue.

FIG. 8-14 showing distribution of lipid in aorta. × 56.

FIG. 8. Arch of aorta from a chick fed basal diet showing absence of lipid staining material.

FIG. 9. Arch of aorta from a chick fed coconut oil and cholesterol showing lipid deposition in the intima.

FIG. 10. Arch of aorta from a chick fed sesame oil and cholesterol showing deposition of lipid in the intima.

FIG. 11. Arch of aorta from a chick fed hydrogenated groundnut oil and cholesterol showing deposition of lipid in the intima.

FIG. 12. Thoracic aorta of a chick fed coconut oil and cholesterol showing similar changes as in Fig. 9.

result in decreased diffusion of nutrients to the intima and the inner half of the media, leading to aggravation of the condition. Increased deposition of collagen in the aorta as evidenced by increased hydroxyproline content in chicks fed cholesterol with oils possibly indicates the existence of a mechanism to strengthen the arterial wall in areas of fragmentation of the elastic tissue.

Histochemical changes: Disruption and degeneration of the elastic tissue and increase in the acid mucopolysaccharides were noticed in the aorta of hypercholesteremic chicks. Intimal proliferation, thickening of intima and complete disappearance of elastic tissue with subsequent production of atheromatous plaques were the changes observed. Increase of acid mucopolysaccharide and decrease of hexosamine in the aorta indicated a reduction of neutral hexosamine containing polysaccharide after the feeding of oils and cholesterol. Deposition of lipid and degeneration of elastic tissue of the aorta were closely related. The elastic tissue changes coupled with disturbances in the metabolism of mucopolysaccharides and collagen might alter the permeability of the arterial wall resulting in formation of atheroma.

The appearance of atherosclerotic lesions in the arch of the aorta earlier than thoracic and abdominal aorta indicated a close similarity in the pattern of development of the condition in man(23). The sites of atheromatosis are also the sites at which stress occurs(24) and the breakdown of elastic tissue is more severe in the region than elsewhere. Therefore the arch of aorta is likely to be more susceptible than other regions of the aorta. Diminished succinic dehydrogenase and succinic oxidase activities were observed in the arch of aorta of hypercholesteremic chickens without any change in the thoracic or abdominal aorta(25). This suggested that metabolic activity of the arch was decreased in atheromatosis.

Results indicated that the severity of the arterial lesion was not parallel to the degree of hypercholesteremia. Although maximum hypercholesteremia was observed in chicks fed hydrogenated groundnut oil and cholesterol, atheromatous lesions were more severe in the arch of aorta of chicks fed sesame oil and chlesterol. The nature of the fat might be responsible for the atheromatous changes observed.

Summary. Portions of aorta of chicks fed coconut oil, sesame oil, mustard oil and hydrogenated groundnut oil, with or without simultaneous administration of cholesterol, for 8 weeks were analyzed for cholesterol, phospholipids, hexosamine and hydroxyproline. The distribution of elastic tissue, acid mucopolysaccharides and lipids in the aorta was also studied by histochemical methods. Feeding of cholesterol with or without oils increased cholesterol and phospholipids in different portions of aorta. Oils when fed alone increased the phospholipids without changing the cholesterol of the aorta. Hexosamine content of the aorta diminished when vegetable oils other than sesame oil were fed either alone or in combination with cholesterol. This indicated a decrease in mucopolysaccharide content of the aorta. Hydroxyproline of aorta increased when chicks were fed vegetable oils with cholesterol. The increase in arterial collagen, as evidenced by its hydroxyproline content, appeared to be proportional to the degree of hypercholesteremia. Feeding of oils with cholesterol increased lipid and

FIG. 13. Thoracic aorta of a chick fed sesame oil and cholesterol showing deposition of lipid in the intima.

FIG. 14. Thoracic aorta of a chick fed hydrogenated groundnut oil and cholesterol showing marked deposition of lipid in intima.

FIG. 15-21 showing distribution of acid mucopolysaccharides in aorta. × 56.

FIG. 15. Arch of aorta of a chick fed basal diet showing slight reaction.

FIG. 16. Arch of aorta of a chick fed coconut oil, increased reaction in intima.

FIG. 17. Same from a chick fed sesame oil and cholesterol, very marked reaction in the intima.

FIG. 18. Same from a chick fed mustard oil and cholesterol, slight reaction.

FIG. 19. Thoracic aorta from a chick fed mustard oil, increased reaction.

FIG. 20. Thoracic aorta from a chick fed mustard oil and cholesterol, increased reaction for mucopolysaccharide in the intima.

FIG. 21. Thoracic aorta from a chick fed hydrogenated groundnut oil and cholesterol, increased reaction.

acid mucopolysaccharide contents of the aorta, proliferation of the intima, and fragmentation or complete disappearance of the elastic tissue in the intima and media. The changes were most marked in the arch of aorta. Elastic tissue changes coupled with disturbances in the metabolism of mucopolysaccharides and collagen might alter the permeability of the arterial wall resulting in atheroma formation. The severity or degree of the different changes was neither related to the plasma level of cholesterol nor to the saturation or unsaturation of the vegetable oils used.

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EFFECT OF MUSTARD OIL (BRASSICA JUNCEA) ON SERUM CHOLESTEROL

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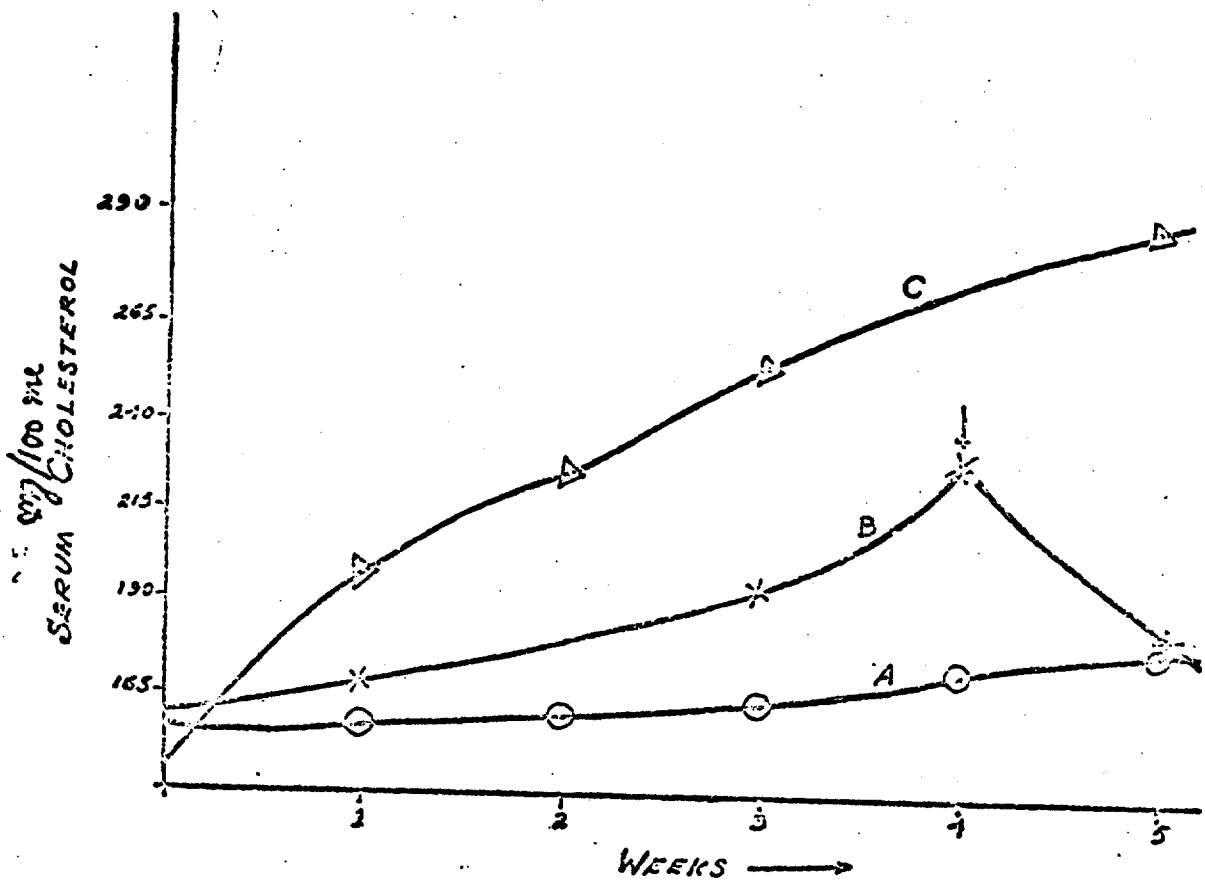
It has come to be almost a common knowledge that coronary heart disease which is responsible for a very large number of death in the economically advanced countries of the west is comparatively less prevalent in the underdeveloped countries (1). Epedemiologic studies of the African Bantu and others (1,2) led special support to this contention.

One of the reasons for this relative absence of coronary heart disease amongst the underdeveloped people is considered as being due to the deficiency of animal fat in the diet of these people (3). Animal fats such as lard or butter Produce hypercholesterolmia (4,5) which is again closely related to the onset of atherosclerosis in the coronary Vessels (5). Certain vegetable oils such as corn oil or sun flower oil bring down the serum cholesterol level. This differential effect of the two kinds of fats has been considered as due to their contents of polyunsaturated fatty acids, the vegetable fat being a richer source of them (6). Recent investigations on the incidence of coronary heart disease in the Indo-Pakistan subcontinent however, indicate that the coronay ailment is not as unfrequent as it is thought to be. Thus, in Northern India, Molhatra and Patharia (7) found that, out of 3054 cardiac cases, 28.4 percent had coronary heart disease. Similar conclusion was also drawn by M. Ibrahim (8) in course of his survey of cardiac cases in East Pakistan. The latter, however, is of opinion that mental worries due to financial insecurity of these people might be the main factor leading to the onset of atherosclerosis.

The vegetable fats used for cooking purposes in this region is mainly mustard oil (Brassica juncea). The investigation described in this paper is concerned with the effect of mustard oil (raw and heated) on the serum cholesterol of rats. It would be seen that mustard oil specially when heated produced a great increase in the serum cholesterol level of the experimental animals. On substitution of the mustard oil, by peanut oil, the cholesterol level went down, as a matter of fact, further than the control.

Experimental

Adult albino rats weighing 160-200 grams raised in this laboratory were used. They were earlier on the general laboratory diet consisting of whole wheat flour, gram, milk and green vegetables. They were placed on a basal diet (Table No. 1) for one week and serum cholesterol determined according to Abell et al (9). The animals were then separated into three groups with 10 animals in each group. Group A were allowed to continue on the basal diet. The experimental fat was incorporated into the basal diet to the level of 5%. The group B animals received the died containing raw mustard oil upto the 4th week. The mustard oil was then replaced by raw peanut oil. The group C animals received the diet with heated mustard oil. The amount of diet for all the animals was so controlled that they were isocaloric and the animals did not put on or lose appreciable weight. Serum cholesterol was determined every week.



A : Control without any fat.
B : Raw Mustard oil, at arrow Mastard oil withdrawn & peanut oil added.
C : Heated mustard oil.

Results & Dicusssion

The results are shown in fig I.

It would be seen that mustard oil increased the serum cholesterol level at the end of the 4th week from 155 mg. to 227 mg./100 ml.i.e. by about 46 percent while the control animals had on increase in the serum cholesterol level by only 9 percent during the period.

The heated mustard oil aggravated the situation further by increasing cholesterol level to almost 93%. Substitution of peanut oil for mustard oil on the 4th week is accompanied with fall of serum cholesterol.

While number of factors such as genetic occupational and environmental etc. may be operating in the etiology of hypercholesterolemeia, it appears to us almost inescapable that mustard oil which is used in almost every home of the east and northern areas of this subcontinent may be a contributory factor towards this condition. While it is not pertinent to recommend at this stage that mustard oil should not be used, we believe that there is justification to explore further the role of this fat in the matter of atherogenesis. It is suspected that allyl isothiocyanate popularly known as *oil of mustard* is the causative factor for increasing cholesterol level. The effect of heating the oil might be due to the destruction of unsaturation and formation of unnatural isomers.

Table 1

	Easal diet for rats
Whole wheat flour	55
Rice bran	15
Mustard oil cake	15
(extracted wiht alcohol)	•
Green vegetables	14
Sodium chloride	1
Calcium carbonate	0.5

Each of the animals also received two drops of codliver oil each week water was supplied at lib.

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EFFECT OF FATS USED IN INDIAN DIET ON CHOLESTEROL AND BLOOD COAGULATION

K. K. Gupta and R. M. Rai.

Recently enough evidence is forthcoming that disturbance in coagulation mechanism plays an integral part in atherogenic process. According to Page[11] atherogenesis is initiated by deposition of fibrin on endothelium. Deposition of layer upon layer of fibrin and overgrown endothelium provide a cushion in which lipid may be deposited. Again Wynn Williams[15] and Montgomery[15] have shown that depletion of chondroitin sulphate B fraction of mucopolysaccharides results in a commensurate loss of anticoagulant property of vessel walls. According to these workers this loss in lipid clearing factor is instrumental in the facilitation of lipid transport through the wall.

Recent work on the thrombogenic theory of atherosclerosis consists in observing the effect of fats on various coagulation tests *in vivo* and *in vitro* or alternatively seeing their effect on fibrinolysin titre of the plasma. On the fats used mostly in Indian diets, the only work in this direction is that of Ramanathan and Gopalan[13], who found that butter, hydrogenated oil and coconut oil produced depression of whole blood clotting time, while the plasma stypvan time was affected by butter only. This finding is not consistent with the findings of O'Brien[10] and Maclagan and Billimoria[7], who found very definite depression of plasma stypvan time in case of butter and no effect on whole blood clotting time or plasma stypvan time by margarine.

As regards effect of fatty meals on fibrinolytic activity, while Greig *et al.*,[5] have shown that post prandial lipaemia inhibits fibrinolysis, Hougie *et al.*,[6] could not confirm these results. No information is available on effects of various kinds of fats on platelet adhesiveness.

In view of these conflicting reports and the fact that many other important oils used in Indian dietary such as mustard, sesame and groundnut oil, were not studied, the present investigations were undertaken to find out:

(a) the relative degree of depression produced in blood coagulation by different fats,

(b) effect of various fats on fibrinogen and fibrinolytic activity of blood,

(c) effect of various fats on platelet adhesiveness,

(d) correlation, if any, of serum cholesterol with above mentioned factors.

METHOD

Fats used in the present experiment are milk, ghee, hydrogenated groundnut oil, groundnut oil, coconut oil, mustard oil and sesame oil. Their iodine value, saturated and unsaturated fatty acids content are shown in Table 1.

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TABLE 1.

Fat	Iodine value	Saturated fatty acid per cent	Monoethenoid fatty acids per cent	Polyethenoid fatty acids per cent
1. Milk Ghee	33	58	38	4
2. Groundnut oil	93	18	56	26
3. Hydrogenated ground-nut oil	63	25	71	4
4. Mustard Oil	103	20	65	25
5. Sesame Oil	115	12	45	43
6. Coconut oil	8	91	7	2

Twenty young adult subjects of age group 20 to 25 years, physically normal in all respects, were selected for this study. They were divided into five groups of four subjects each and one group each day from Monday to Friday, was studied. One type of fat was fed each week to all the 20 subjects, thus six fats were used in six weeks during which the experiment lasted. In the morning, sample of fasting blood was collected in an ordinary glass syringe. Then the subjects were fed on a breakfast consisting of 150 g. atta (wheat) in the form of chappaties and 65 g. of the fat under study cooked with vegetable (potatoes 56 g., onions 28 g., and green vegetable 56 g.). Four hours after breakfast, a second sample of blood was collected.

The coagulation tests done were as follows:

1. Whole blood clotting time—The method of Lee & White[8] was followed.

2. Plasma stypvan time—Plasma was obtained by centrifuging citrated blood (one part of 3.8 per cent sodium citrate in water to 9 parts of blood) at 1,500 r.p.m. for 15 minutes. To 0.1 cc. plasma and 0.1 cc. of 1: 10,000 Russell's viper venom (stypvan) was added 0.1 cc. of M/40 calcium chloride a minute later. The test was repeated twice, and the clotting times were averaged.

3. Fibrinolytic activity was measured by the method of partial lysis as described by Bidwet[2].

4. Platelet adhesiveness was done by the technique described by Payling Wright[12].

Total serum cholesterol was also estimated in the pre- and post-prandial samples in each case by the method of Abell *et al.*[1]

RESULTS

Means of the results of various tests done during fasting and four hours after feeding various fats are shown in Tables 2 to 7.

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TABLE 2—*Means of results during fasting and 4 hours after meals with milk ghee (Clarified butter)*

Test	Fasting	4 hrs after fatty meals	Significance of difference
Whole Blood Clotting Time in Seconds	226.66 ±6.59	209.99 ±10.17	N.S.
Stypvan time of Plasma in Seconds	29.55 ±1.68	21.48 ±2.20	P∠.01
Platelet Count per cmm	3,44,000	3,17,000	N.S.
Platelet Adhesiveness after ½ hour	45.3%	61.2%	P∠.01
1 hour	61.3%	69.7%	P∠.05
1½ hours	70.3%	67.8%	N.S.
2 hours	76.8%	85.2%	P∠.05
Fibrinogen Content (mg.%)	182.6	182.6	N.S.
Total lysis fibrin in 3 hours (mg.%)	30	14	P∠.01
Cholesterol (mg. %)	160.18	167.36	P∠.01

TABLE 3—*Mean of results during fasting and 4 hours after fatty meals with groundnut oil*

Test	Fasting	4 hours after fatty meals	Significance of difference
Whole Blood Clotting Time in Seconds	230.88 ±7.39	190.23 ±12.93	P∠.05
Stypvan Time of Plasma in Seconds	34.38 ±1.68	27.78 ±1.75	P∠.01
Platelet Count per cmm	3,30,000	3,46,000	N.S.
Platelet Adhesiveness after ½ hour	47%	45.3%	N.S.
1 hour	60%	52.9%	P∠.05
1½ hours	68.2%	61.3%	P∠.05
2 hours	73.7%	69.4%	N.S.
Fibrinogen Content (mg. %)	175	171.6	N.S.
Total lysis fibrin in 3 hrs (mg. %)	2.4	8.4	P∠.05
Cholesterol (mg. %)	148.93	149.80	N.S.

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TABLE 4—*Mean of results during fasting and 4 hours after fatty meals with hydrogenated groundnut oil*

Test	Fasting	4 hours after fatty meals	Significance of difference
Whole Blood Clotting Time in Seconds	205.50 ±4.16	182.05 ±5.09	P∠.01
Stypvan time of Plasma in Seconds	28.9 ±1.19	25.94 ±1.20	P∠.05
Platelet Count per cmm	3,23,000	3,96,000	N.S.
Platelet Adhesiveness after			
½ hour	31.3%	65.2%	P∠.01
1 hour	57.0%	73.5%	P∠.01
1½ hours	69.4%	76.9%	P∠.05
2 hours	64.6%	79.4%	P∠.05
Fibrinogen Content (mg. %)	136.3	141.5	N.S.
Total lysis fibrin in 3 hours (mg. %)	17.0	11.2	P∠.05
Cholesterol (mg. %)	149.85	156.46	P∠.01

TABLE 5—*Mean of results during fasting and 4 hours after fatty meals with coconut oil*

Test	Fasting	4 hours after fatty meals	Significance of difference
Whole Blood Clotting Time in Seconds	241.84 ±6.97	233.82 ±8.02	P∠.05
Stypvan Time of Plasma in Seconds	31.06 ±3.97	25.32 ±1.26	P∠.01
Platelet Count per cmm	3,92,000	2,91,000	N.S.
Platelet Adhesiveness after			
½ hour	70.7%	40.3%	P∠.01
1 hour	72.2%	41.3%	P∠.01
1½ hours	79.9%	60.9%	P∠.05
2 hours	80.9%	65.3%	P∠.05
Fibrinogen Content (mg. %)	166.6	185.7	N.S.
Total lysis fibrin in 3 hrs (mg. %)	11.2	20.2	P∠.05
Cholesterol (mg. %)	153.86	162.23	P∠.01

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TABLE 6—*Mean of results during fasting and 4 hours after fatty meals with mustard oil*

Test	Fasting	4 hours after fatty meals	Significance of difference
Whole Blood Clotting Time in Seconds	213.32 ±7.89	199.48 ±5.38	P∠.01
Stypvan Time of Plasma in Seconds	28.16 ±1.02	25.37 ±1.07	N.S.
Platelet Count per cmm	2,58,000	2,54,000	N.S.
Platelet Adhesiveness after			
½ hour	18.6%	26%	N.S.
1 hour	37.2%	41.9%	N.S.
1½ hours	51.0%	48.0%	N.S.
2 hours	63.6%	51.0%	P∠.01
Fibrinogen Content (mg.%)	200	190	N.S.
Total lysis fibrin in 3 hours (mg.%)	80	79	N.S.
Cholesterol (mg.%)	152.08	152.30	N.S.

TABLE 7—*Mean of results during fasting and 4 hours after fatty meals with seasame oil*

Test	Fasting	4 hours after fatty meals	Significance of difference
Whole Blood Clotting Time in Seconds	223.94 ±6.50	183.15 ±5.25	P∠.01
Stypvan Time of Plasma in Seconds	26.92 ±0.91	23.89 ±0.92	P∠.01
Platelet Count per cmm	3,22,000	2,70,000	P∠.05
Platelet Adhesiveness after			
½ hour	8.7%	8.7%	N.S.
1 hour	36%	32.6%	N.S.
1½ hours	54%	50.4%	N.S.
2 hours	64.6%	54.9%	P∠.01
Fibrinogen Content (mg%)	193.9	187.8	N.S.
Total lysis fibrin in 3 hours (mg %)	29.1	66.7	P∠.01
Cholesterol (mg %)	150.6	149.31	N.S.

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DISCUSSION

Whole Blood Clotting Time and Plasma Stypvan Time

The results of this study show that all the fats significantly depress whole blood clotting time. Stypvan time of plasma is shortened significantly by all fats except mustard oil. As regards the differential response of these fats to different tests, it is observed that ghee (clarified butter) has a tendency to depress stypvan time of plasma more significantly than mustard oil and sesame oil. In other results there is no significant difference in the response of various fats.

Fibrinolysin Titre and Fibrinogen Content

The fibrinolysin property of blood was depressed by milk ghee (clarified butter), hydrogenated groundnut oil while it was increased by vegetable oils such as groundnut oil, sesame oil and coconut oil. Mustard oil however, showed no significant change. These results are not in agreement with those of Farquhar *et al.*,[4] who showed that fibrinolysin was equally inhibited by prior ingestion of equal amounts of either a relatively saturated animal fat (butter fat) or highly unsaturated fat (safflower oil). Our results differ from those of Hougie *et al.*,[6] who found no effect of lipaemia on fibrinolysin titre. Our results support the view of Greig *et al.*,[5] who have shown that there is marked influence on fibrinolysin by creams, while after vegetable oils fibrinolysin rose above the fasting level.

As regards fibrinogen content, milk ghee (clarified butter) showed no difference, while hydrogenated groundnut oil and coconut oil showed an increase. Unsaturated vegetable oils such as mustard oil and sesame oil lessened the fibrinogen content. The differences in each case were on the verge of significance. It appears that there is an inverse relationship between fibrinogen content and fibrinolysin titre. Our results except in case of coconut oil generally support this contention.

Platelet Count and Platelet Adhesiveness

No significant change in platelet count was produced by any of the fats. Platelet adhesiveness was increased significantly by milk ghee and hydrogenated groundnut oil, while groundnut oil, mustard oil, sesame oil and coconut oil markedly decrease the adhesiveness of the platelets. These results assume importance as according to McDonald[9], Cullen and Swank[3] increased platelet adhesiveness is the best indication of intravascular hypercoagulability.

Serum Cholesterol

Saturated fats such as milk ghee (clarified butter), hydrogenated groundnut oil, coconut oil increased serum cholesterol significantly, while, unsaturated

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vegetables such as mustard oil and sesame oil and groundnut oil showed a tendency of reducing it. With increase of cholesterol, platelet adhesiveness generally increased and fibrinolysin titre decreased and reverse tendency was generally seen with oil showing hypocholesterolaemic property. McDonald and Edgill[8] showed diminished platelet adhesiveness and serum cholesterol on rice fruit diet but according to him change in stickiness was not due to reduction in cholesterol.

Coconut oil deserves special mention. In some tests it has property of saturated animal fats such as increase of cholesterol and fibrinogen content, while in others it resembles unsaturated vegetable oils, as like them it increases fibrinolysin titre and decreases platelet adhesiveness. Why coconut oil behaves like animal fats in some respects and like unsaturated vegetable oils in others is rather difficult to explain. Short chain fatty acids in this saturated oil may be responsible for its differential effects.

In conclusion we may say that some of our results require further confirmation and in case found true they will demand explanations which may not be so easily forthcoming.

SUMMARY

The effect of ghee (clarified butter), groundnut oil, hydrogenated groundnut oil, coconut oil, mustard oil and sesame oil in blood coagulation of twenty human volunteers has been studied. The main findings are as follows:

(a) All the fats produced significant depression of whole blood clotting time.

(b) Stypvan time of plasma was depressed by all fats except mustard oil.

(c) Clarified butter (ghee) and hydrogenated groundnut oil increased the platelet adhesiveness while groundnut oil and coconut oil and sesame oil decreased it. As regards mustard oil significant decrease in platelet adhesiveness was seen only after two hours.

(d) Fibrinolysing property of blood was decreased by clarified butter and hydrogenated groundnut oil while it was increased by groundnut oil, coconut oil and sesame oil. Mustard oil showed no effect on fibrinolysin titre.

(e) Hydrogenated oil and coconut oil showed a trend to increase the fibrinogen content and mustard oil and sesame oil to diminish it. Other fats showed no effect.

(f) No change in platelet count was seen by any of the fats studied.

(g) Milk ghee, hydrogenated groundnut oil caused a significant increase in serum cholesterol while other oils had little effect.

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STUDY OF CHEMICAL REPRESENTATIVES OF THE GOITROGENIC ACTIVITY OF RAW CABBAGE

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In the preceding experiments (Langer and Štole 1964b) the goitrogenic activity of cabbage was demonstrated in rats by modern methods and older observations by other authors (reviewed by Greer 1950, 1964) were confirmed.

Analytical methods were then elaborated for determining various natural compounds assumed to possess goitrogenic activity (in particular isothiocyanates and 2-thiooxazolidones) and the conditions of rat experiments were modified, making it possible to analyse the individual substances in cabbage consumed by rats and to determine the effect of the same doses of the pure substances on the rat thyroid. The results of these experiments are given below.

Material and Methods

1. *Animals and diet:* 139 white male rats (Wistar offspring, Dobrá Voda breed) with an initial weight of 110–130 g were used. Two experiments (A – 119 rats and B – 20 rats) were carried out. Up to the start of the experiment, the animals were fed a Larsen diet, with a daily iodine intake of 2 μg iodine/rat. At the start of experiment A, 15 rats were killed (group 1) and the rest were put on to a low iodine diet elaborated in the author's laboratory. It consisted of a dry mixture containing wheat gluten (17.5%), dried brewer's yeast (20%), margarine (10%), starch (49.5%), a salt mixture (Srinivasan et al. 1957) (3%) and mixed vitamins (Langer, Štole and Kutka 1964). With this diet the daily iodine intake fell to 0.3–0.5 μg/rat. On the tenth day of experiment A, 11 rats were killed (group 2). The others were then divided into ten groups, all of which received the low iodine diet (LID) and carrot (Crr) or cabbage (Cbb) and various other substances which were always administered, in the amounts given below, in 3 ml distilled water per rat by stomach tube:

groups 3–I and 3–II	LID + Crr
groups 4–I and 4–II	LID + Crr + 3 ml distilled water
groups 5–I and 5–II	LID + Cbb
groups 6–I and 6–II	LID + Crr + 1 mg thiocyanate (as KSCN)
group 7–I	LID + Crr + 6 mg AITC[1]/
group 7–II	LID + Crr + 2 mg AITC[1]/

The groups with the number I were killed after 20 more days and those with the number II after 50 days. In this experiment the animals were given no drinking water. They received the necessary amount of water in the form of vegetables, of which they consumed about 25 g daily per rat (Tab. 2). As a result, their basic diet intake, and hence their calorie intake, was lower.

Experiment B was started 31 days after experiment A, with rats of the same initial group, whose body weight was meantime somewhat higher, however, because of their higher caloric intake.

[1]) AITC = allylisothiocyanate
[2]) MTO = L–5,5'-dimethyl-2-thiooxazolidone
[3]) SCN⁻ = thiocyanate

As in experiment A, the animals were all fed first of all on a low iodine diet for 10 days and were then divided into three groups:

group 8	LID + Crr
group 9	LID + Crr + 100 μg MTO[2]/
group 10	LID + Crr + a mixture of 0.5 mg SCN[-]/, 0.5 mg ATC[1]/ and 100 μg MTO[2]/

After 20 more days the animals were killed.

2. *Determination of diet and vegetable consumption.* The animals were kept in cages in groups of 4–7. The amount of vegetable and diet was weighed daily for each cage and the next day the scraps were carefully collected and weighed. At the end of the experiment, the average diet and vegetable consumption/rat/day was calculated for every group. Every day, the food allocation was based on the cage with the lowest consumption on the previous day. This ensured the same average food consumption in all groups of rats in the relevant time interval of the same experiment (Tab. 2).

3. *Determination of iodine intake.* Each batch of diet, and the cabbage and carrots used in the experiment, were analysed for the iodine content by Štole's method (Štole 1963a). The diet contained 2.7–3.4 μg iodine/100 g, the cabbage 0.6–0.9 μg and the carrots 0.5–0.8 μg/100 g wet weight. The average daily iodine intake was calculated from the known diet and vegetable consumption and from the amount of iodine which these were known to contain (Tab. 2).

4. *Killing the animals and determination of thyroid function.* The methods (determination of thyroid weight, the accumulation of ^{131}I, the ^{127}I content and the PBI level) were described in the preceding paper (Langer and Štole 1964b).

5. *Determination of thiocyanate, mustard oils and L-5-vinyl-2-thiooxazolidone in cabbage.* Winter cabbage (*Brassica oleraceae*, var. *Capitata*, subspec. Amager, harvested in 1963 and stored up to the end of the experiment (March 24, 1964) at 0–5 °C, was used. The thiocyanate concentration was determined in every head of cabbage used for feeding the animals by Michailovsky and Langer's modification (1958) of Aldridge's method (1944). The mustard oils and L-5-vinyl-2-thiooxazolidone concentration were determined by a method elaborated in the author's laboratory (awaiting publication).

Intact cabbage leaves were weighed and thrown into boiling alcohol. After boiling for 5 min and cooling, the mixture was homogenized in a kitchen mixer and filtered. The methyl alcohol was then evaporated off in vacuo at 50 °C and the plant extract residue was weighed. An aliquot volume of plant extract (usually 0.1 ml, corresponding to 0.3–0.6 g of the original cabbage) was added to 0.2 M phosphate buffer at pH with traces of ascorbic acid, up to 0.9 ml volume. The mixture was extracted three times with 3 ml ether, thus removing most of the interfering substances extractable by ether (first extraction); 0.1 ml myrosinase solution (Neuberg and Wagner 1926) was then added for enzymatic decomposition of thioglycosides (two hours at room temperature). Mustard oils and L-5-vinyl-2-thiooxazolidone (VTO) were then extracted three times with a double volume of ether (second extraction). After this second extraction, the total and individual mustard oil concentration and the VTO concentration were determined by different techniques.

Total mustard oils were determined by adding ammonia to the second extract, which was followed by their conversion to the corresponding thiourea derivatives. After evaporating the ether and ammonia to dry weight, the extract was dissolved in a further volume of ether and was measured photometrically against ether in the UV zone on a CF 4 (Optica, Milano) spectrophotometer in the 230–270 mμ range. A sharp maximum was usually found at 250 mμ; this is in agreement with the wavelength of the absorption maximum of different monosubstituted thioureas in ether. Average extinction at 240 and 260 mμ and the final extinction of the non-volatile fraction (v. below) were subtracted from extinction at 250 mμ. The thiourea concentration was read off by means of this final extinction calculation from the calibration curve of allyl thiourea in ether, and after stoichiometric conversion the total mustard oils content was expressed as the allyl isothiocyanate concentration/100 g of the original plant material.

VTO was determined in the non-volatile fraction of the second extract, which was evaporated until dry by means of an infra-red lamp; ether was then added and again evaporated off. This removed all the volatile isothiocyanates and the extract contained mainly VTO, sulphoraphan and one unidentified substance. When measuring the ultraviolet absorption spectrum in ether, its non-volatile fraction gave maximum absorption at 248 mμ; this is in agreement with the wavelength of maximum absorption for VTO in ether. Average extinction at 236 and 260 mμ was substracted from maximum extinction and the final concentration was read off from the calibration curve of VTO in ether. The substances present in the non-volatile part of ether extract were expressed as the VTO concentration in 100 g of the original plant material. As seen from the above findings, expressed in this manner the result was somewhat higher than the actual VTO concentration.

When determining the percentage proportion of individual mustard oils, the second ether

extract was first distilled off, giving a relatively very pure mustard oils mixture almost free of interfering substances. Ammonia was added to the distillate, giving rise to the corresponding thiourea derivatives. After evaporating until dry, the extract was dissolved in a small amount of ethanol and aliquot volumes were applied to Whatman No. 1 chromatographic paper. The chromatograms were developed in a water-saturated chloroform system. Thiourea spots were detected by means of a low-pressure mercury vapour lamp and were then twice eluted with 3 ml hot ethanol. In the ultraviolet zone, the eluates gave maximum absorption at 243 mμ (measured against ethanol). Average extinction at 230 and 256 mμ was substracted from this extinction and the sum of the final extinctions of all the spots from the same specimen was regarded as 100%. From this, the percentage proportion of the individual substances was calculated.

Results

A. Basic observations

1. Concentration of some natural antithyroid substances in cabbage. As seen from Tab. 1, the concentration of these substances varied within a relatively wide range of values. In some cases (14–18, 20, 23, 24), the cabbage contained high concentrations of all the given sulphur compounds, while in others the concentration of all these substances was low (1, 2, 6, 7, 9, 13). Of the mustard oils, allyl isothiocyanate was present in by far the largest amounts. The concentration of the others was much lower and for the calculations in this experiment it was virtually negligible.

2. Food intake, body weight and growth curves. In the same time interval of the same experiment, food intake was always practically the same (Tab. 2). This means that the food volume in all the groups was also the same—an important finding from the aspect of the dynamics of the enterohepatic cycle of the thyroid hormones (van Middlesworth 1960). The body weight in all groups was also practically the same at the same time interval of the same experiment (Tab. 2); this also applies to the growth curves (not illustrated). The only exception were rats fed on cabbage for 50 days (group 5-II), which despite slightly higher food consumption gained weight much more slowly than the other animals and during the last three weeks actually lost weight. This is attributed to raised thyroid hormone release induced by TSH. In general, these animals were more irritable and aggressive than the others and they also had tachycardia.

3. Iodine intake. Because the animals had the same diet and vegetable intake, with the same iodine content, the iodine intake of all the groups was also the same. In absolute values it was 0.3–0.5 μg/day/rat. Under these conditions of iodine deficiency, the changes in thyroid function observed in the various experimental groups can be attributed almost entirely to the antithyroid effect of the given test substances.

4. Intake of individual natural antithyroid substances. The intake of thiocyanate, allyl isothiocyanate (total mustard oils) and L-5-vinyl-2-thiooxazolidone (L-5,5'-dimethyl-2-thiooxazolidone) in the individual groups is given in Tab. 2.

B. Effect of individual test factors on the thyroid

1. Effect of iodine deficiency. This was studied progressively in the control groups of both experiments (Tab. 2). The weight of the thyroid underwent a pronounced progressive increase, with statistically highly significant differences between the individual time intervals. Thyroid radioactivity remained within the same limits throughout the experiment. No particular importance is attached to the finding that on one occasion it was significantly higher than on the two preceding intervals and on another significantly lower. Experience has shown that this may be due to

l groups of rats

	^{127}I in thyr	
	μg M ± S.D.	P
	3.93 ± 1.07	—
	3.08 ± 0.85	0.0
	0.53 ± 0.23	0.0
	1.04 ± 0.33	—
	0.79 ± 0.29	—
	0.83 ± 0.32	—
	0.85 ± 0.34	—
	1.10 ± 0.58	—
	0.92 ± 0.29	—
	0.59 ± 0.22	NS
	1.31 ± 0.35	—
	0.75 ± 0.12	—
	0.90 ± 0.36	—

Table I

Concentration of individual antithyroid substances in cabbage used to feed animals in experiment A. VTO 1,-5-vinyl-2-thiooxazolidone, AITC allyl isothiocyanate, 3-BITC 3-butenyl isothiocyanate, n-BITC butyl isothiocyanate, X_1, X_2 unidentified substances

Cabbage No.	Thiocyanate mg SCN/100 g	Non-volatile substances µg VTO/100 g	Volatile mustard oils mg AITC/100 g	Percentage of individual volatile mustard oils in distillate				
				AITC	3-BITC	X_1	n-BITC	X_2
1	1.30	50	0.37	100	—	—	—	—
2	1.25	50	0.73	100	—	—	—	—
3	1.40	550	1.73	65	14	—	9	12
4	1.65	50	1.39	100	—	—	—	—
5	2.85	50	0.36	100	—	—	—	—
6	0.90	50	0.63	100	—	—	—	—
7	1.15	50	0.82	100	—	—	—	—
8	1.70	500	2.10	100	—	—	—	—
9	1.55	50	0.93	100	—	—	—	—
10	5.70	50	6.93	100	—	—	—	—
11	2.25	50	4.71	100	—	—	—	—
12	1.05	50	2.87	100	—	—	—	—
13	1.05	50	1.94	100	—	—	—	—
14	3.40	270	8.06	79	5	5	5	6
15	6.15	470	20.19	91	3	3	—	3
16	6.80	950	7.30	86	5	3	3	3
17	4.90	110	2.52	100	—	—	—	—
18	2.05	250	2.47	100	—	—	—	—
19	5.12	50	4.48	91	—	4	5	—
20	1.76	1917	8.82	76	3	—	17	4
21	1.58	160	4.76	100	—	—	—	—
22	0.97	80	4.49	100	—	—	—	—
23	2.31	740	6.62	88	9	3	—	—
24	1.95	1245	3.65	55	4	—	3	38
Total	59.88	7852	97.97					
Average	2.49	327	4.08					
Minimum	0.9	50	0.36					
Maximum	6.8	1917	20.19					

the great variability of this indicator. The total thyroid iodine concentration and the serum protein-bound-iodine fell highly significantly, especially at the outset of the experiment. The serum thiocyanate level was very low. No marked differences in any of the given indicators were found between control groups 3 and 4 (not given) and these two groups were therefore excluded. They show that the administration of 3 ml. distilled water/day/rat by stomach tube and the manipulation associated

Table 2

Intake of low iodine diet (LID) and individual antithyroid substances and indicators of thyroid function in individual groups of rats

Body weight g. M±S. D	Daily intake of diet, vegetables and antithyroid substances (per rat)					Weight of thyroid			^{131}I in thyroid			^{127}I in thyroid			PBI in serum			SCN⁻ in serum	
	LID g	vegetables g	SCN⁻ mg	total mustard oils mg AITC	VTO² (MTO³) µg	mg 100 g M±.S. D.	Statistical significance P_1^4	Statistical significance P_2^5	Percentage of dose M±S.D.	statistical significance P_1^4	statistical significance P_2^5	µg M±S.D.	statistical significance P_1^4	statistical significance P_2^5	µg % M±S.D.	statistical significance P_1^4	statistical significance P_2^5	mg % M±S.D.	statistical significance P_2^5
124±12	—	—	—	—	—	5.44±1.37	—	—	24.91± 6.34	—	—	3.93±1.07	—	—	2.47±1.20	—	—	—	—
133± 9	10,7	—	—	—	—	8.29±1.70	0.001	—	34.14± 6.57	0.01	—	3.08±0.85	0.05	—	1.46±0.32	0.02	—	0.29±0.07	—
141±25	6.0	22	—	—	—	11.46±2.42	0.001	—	26.72± 7.51	0.05	—	0.53±0.23	0.001	—	0.89±0.31	0.001	—	0.11±0.03	—
136±12	5.8	26	0.42	0.30	39	15.58±4.96	—	0.05	39.38± 6.57	—	0.01	1.04±0.33	—	0.01	1.18±0.46	—	NS	0.98±0.26	0.001
144±32	5.7	22	1.00	—	—	13.92±3.51	—		39.80±11.19	—	0.02	0.79±0.29	—	NS	0.68±0.08	—	NS	0.72±0.12	0.001
141±15	5,2	24	—	6.00	—	15.02±4.73	—	0.05	35.21± 7.91	—	NS	0.83±0.32	—	0.05	0.57±0.31	—	NS	0.61±0.08	0.001
171±18	6.9	28	—	—	—	10.74±3.27	—	—	24.16± 8.22	—	—	0.85±0.34	—	—	0.98±0.49	—	—	0.08±0.02	—
173±15	7.2	27	—	—	100	11.57±1.54	—	NS	27.87± 9.99	—	NS	1.10±0.58	—	NS	1.10±0.50	—	NS	0.14±0.05	NS
164±22	6.8	25	0.50	0.50	50	16.13±3.10	—	0.02	32.26± 7.41	—	NS	0.92±0.29	—	NS	0.66±0.17	—	NS	0.66±0.06	0.001
164±20	6.5	26	—	—	—	15.99±3.24	0.001	—	28.46± 9.94	NS	—	0.59±0.22	NS	—	0.62±0.25	NS	—	0.15±0.05	—
138±13	7.1	27	0.56	0.93	55	27.24±6.21	—	0.001	46.35± 8.14	—	0.001	1.31±0.35	—	0,05	0.39±0.23	—	0,01	0.83±0.30	0.001
167± 9	6.4	25	1.00	—	—	22.46±9.16	—	0.01	28.01± 5.52	—	NS	0.75±0.12	—	NS	0.59±0.27	—	NS	0.65±0.15	0.001
163±30	6.4	26	—	2.00	—	15.88±4.08	—	NS	33.77±11.43	—	NS	0.90±0.36	—	NS	0.60±0.24	—	NS	0.52±0.21	0.001

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Table 2

Intake of low iodine diet (LID) and individual antithyroid substances and indicators of thyroid function in individual groups of rats

Experiment	Duration of experiment: LID days	Duration of experiment: LID vegetables days	Group: Group No.	Group: Substances administered	Number of rats	Body weight g. M±S. D	Daily intake of diet, vegetables and antithyroid substances (per rat): LID g	vegetables g	SCN⁻ mg	total mustard oils mg AITC	VTO[2] (MTO[3]) μg	Weight of thyroid: mg 100 g M±.S. D.	Statistical significance: P_1^4	Statistical significance: P_2^5	^{131}I in thyroid: Percentage of dose M±S.D.	statistical significance: P_1^4	statistical significance: P_2^5	^{127}I in thyroid: μg M±S.D.	statistical significance: P_1^4	statistical significance: P_2^5
A	0	0	1	control	15	124±12	—	—	—	—	—	5.44±1.37	—	—	24.91± 6.34	—	—	3.93±1.07	—	—
A	10	—	2	control	11	133± 9	10,7	—	—	—	—	8.29±1.70	0.001	—	34.14± 6.57	0.01	—	3.08±0.85	0.05	—
A	10	20	3+4–I	control	13	141±25	6.0	22	—	—	—	11.46±2.42	0.001	—	26.72± 7.51	0.05	—	0.53±0.23	0.001	—
			5–I	cabbage	6	136±12	5.8	26	0.42	0.30	39	15.58±4.96	—	0.05	39.38± 6.57	—	0.01	1.04±0.33	—	0.01
			6–I	SCN⁻	5	144±32	5.7	22	1.00	—	—	13.92±3.51	—		39.80±11.19	—	0.02	0.79±0.29	—	NS
			7–I	AITC[1]	6	141±15	5,2	24	—	6.00	—	15.02±4.73	—	0.05	35.21± 7.91	—	NS	0.83±0.32	—	0.05
B	10	20	8	control	6	171±18	6.9	28	—	—	—	10.74±3.27	—	—	24.16± 8.22	—	—	0.85±0.34	—	—
			9	MTO[3]	7	173±15	7.2	27	—	—	100	11.57±1.54	—	NS	27.87± 9.99	—	NS	1.10±0.58	—	NS
			10	SCN⁻+AITC[1] +MTO[3]	7	164±22	6.8	25	0.50	0.50	50	16.13±3.10	—	0.02	32.26± 7.41	—	NS	0.92±0.29	—	NS
A	10	50	3+4–II	control	28	164±20	6.5	26	—	—	—	15.99±3.24	0.001	—	28.46± 9.94	NS	—	0.59±0.22	NS	—
			5–II	cabbage	13	138±13	7.1	27	0.56	0.93	55	27.24±6.21	—	0.001	46.35± 8.14	—	0.001	1.31±0.35	—	0,05
			6–II	SCN⁻	11	167± 9	6.4	25	1.00	—	—	22.46±9.16	—	0.01	28.01± 5.52	—	NS	0.75±0.12	—	NS
			7–II	AITC[1]	11	163±30	6.4	26	—	2.00	—	15.88±4.08	—	NS	33.77±11.43	—	NS	0.90±0.36	—	NS

- AITC = allyl isothiocyanate
- VTO = L-5-vinyl-2-thiooxazolidone
- MTO = L-5,5'-dimethyl-2-thiooxazolidone
- P_1 = statistical significance of comparison of the corresponding control group (by Student's t-test) with the control group in the preceding interval of experiment A
- P_2 = statistical significance of comparison of the corresponding group (by Student's t-test) with the control group for the same experiment and time interval

with this treatment does not affect the given indicators of thyroid function. The values in all the control groups in experiments A and B after 20 days were also the same, showing that the conditions in both experiments were reproducible and capable of comparison. A low iodine intake, therefore, has marked goitrogenic effects.

2. Effect of cabbage. The weight of the thyroid after 20 and 50 days was significantly higher than in the two control groups (Tab. 2). Thyroid radioactivity and total iodine were also significantly raised at both intervals, probably as a result of an increase in the ability of the thyroid to accumulate iodine. The significant decrease in serum protein-bound-iodine (after 50 days) is in agreement with previous findings (Langer et al. 1964, Langer and Štolc 1964a, b). The serum thiocyanate level was highly significantly raised as compared with the control groups. It was also (nonsignificantly) higher than in the other groups.

With a low iodine intake, therefore, cabbage had a much stronger goitrogenic effect than iodine deficiency alone.

3. Effect of thiocyanate. In both groups (6-I and 6-II), the weight of the thyroid increased, the increase after 50 days being statistically significant as compared with the control groups. After 20 days there was also a significant increase in thyroid radioactivity. Total thyroid iodine and serum protein-bound-iodine did not differ significantly from the controls in either group. The serum thiocyanate level was highly significantly raised as compared with the control groups, but it is interesting to note that at both time intervals it was distinctly, though non-significantly, lower than in the cabbage groups.

Evaluation of the effect of thiocyanate on the weight of the thyroid shows that it was about half that of cabbage at both intervals. The dose of thiocyanate in these groups, however, was roughly double the amount consumed in cabbage. The same dose of thiocyanate as that consumed in cabbage would therefore have about 20–30% the goitrogenic effect of cabbage. This is in agreement with the results of previous experiments (Langer and Štolc 1964a). It is nevertheless interesting that a relatively small dose of SCN^- (with a relatively low serum level) had a pronounced goitrogenic effect under the given conditions.

4. Effect of allyl isothyiocyanate. In group 7-I, which was given 6 mg AITC/rat/day (about 20 times higher than the total mustard oils intake determined in cabbage in group 5-I), the weight of the thyroid was statistically significantly higher than in the control groups. The radioactivity of the thyroid was non-significantly higher (Tab. 2). Total thyroid iodine increased significantly. Serum protein-bound-iodine did not differ significantly from the control values. The serum thiocyanate level was highly significantly raised, showing that AITC was metabolized in the organism to thiocyanate.

In group 7-II, to which 2 mg. AITC/rat/day was administered, no statistically significant difference was found after 50 days as compared with the control groups, apart from a raised serum thiocyanate level.

Large doses of allyl isothiocyanate thus have a goitrogenic effect (in agreement with previous findings—Langer and Štolc 1964c). In a dose about 10–20 times larger than the total mustard oil intake in group 5-I (cabbage fed), its effect was very similar to that of cabbage, while in an approximately double dose it had no effect.

5. Effect of L-5,5'-dimethyl-2-thiooxazolidone. As seen from Tab. 2, a double dose of this substance as compared with the L-5-vinyl-2-thiooxyzolidone intake in the cabbage group had no demonstrable effect on the thyroid. Because of the very limited amount of this substance available, it could not be administered either

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in large doses or for long periods to several animals. Because of its very similar chemical structure, MTO ought, theoretically, to have the same goitrogenic activity as L-5-vinyl-2-thiooxazolidone (Iino 1961) and L-5-phenyl-2-thiooxazolidone (Greer and Whallon 1961, Krusius and Pelola 1964), although its activity has not previously been tested directly.

6. Effect of a mixture of thiocyanate, allyl isothiocyanate and L-5,5'-dimethyl-2-thiooxazolidone. Group 10 in experiment B was given almost the same doses of thiocyanate, allyl isothiocyanate (as compared with total mustard oil intake) and L-5,5'-dimethyl-2-thioxazolidone (as compared with L-5-vinyl-2-thiooxazolidone intake) as the cabbage fed group. As seen from Tab. 2, the weight of the thyroid in this group was significantly higher than in the control and the same as after cabbage. Thyroid radioactivity was nonsignificantly raised. Total thyroid iodine and serum protein-bound-iodine did not differ significantly from the control. In general, changes in the thyroid (especially its weight) were very similar to changes observed after 20 days cabbage feeding (group 5-I).

Since the individual administration of several times larger doses of these substances was either ineffective (L-5,5'-dimethyl-2-thiooxazolidone in group 9 and allyl isothiocaynate in group 7-II) or much less effective (thiocyanate in groups 6-I and 6-II), it must be concluded that when administered together, even in small doses, they potentiate each other's effect. On the basis of the present findings, the possibility that the mixture of analogous natural substances present in cabbage acts in a similar manner should be considered.

Discussion

Identical food consumption and an identical low iodine intake, based on identical composition of the food in all groups of rats, formed the basic conditions for an exact evaluation of the effect of various substances on the thyroid. Another important technical factor was quantitative determination of the concentration of individual natural antithyroid substances in cabbage consumed by the rats, which made it possible to select aliquot doses of the pure substances and test their quantitative relationship to the doses of the same substances consumed in cabbage.

This approach facilitated evaluation of the approximate part played by the individual substances in the goitrogenic effect of cabbage. The most remarkable of these substances was thiocyanate (formed by decomposition of the thioglucoside sinigrin—Gmelin and Virtanen 1960), which was the only one which produced thyroid hypertrophy and some of the changes in other indicators in doses similar to those in cabbage. Quantitatively analogous doses of L-5,5'-dimethyl-2-thiooxazolidone and allyl isothiocyanate to those of L-5-vinyl-2-thiooxazolidone and total mustard oils consumed in cabbage had no such effect. The effect of thiocyanate by itself means completely explains the goitrogenic effect of cabbage, however, while effective doses of allyl isothiocyanate are much larger than its concentration in cabbage.

The simultaneous administration of thiocyanate, allyl isothiocyanate and L-5,5'-dimethyl-2-thiooxazolidone in the same doses in which these, or structurally closely analogous substances, are found in cabbage, had a remarkable effect. Their effect on the thyroid was very similar to that of cabbage. With reference to present knowledge on biosynthesis of the thyroid hormones, it is admissible to assume that they act as synergists. Thiocyanate is known to interfere in the incorporation of iodide, i.e. in the initial phase of biogenesis (Vanderlaan and Vanderlaan 1947) and it can

also interfere in other phases (Vlijm 1958, Štole 1963b). The effect of L-5-vinyl-2-thiooxazolidone is similar to that of thiourea and thiouracil type substances and it interferes mainly in iodide oxidation and the organic binding of iodine (Iino 1961). The mechanism of the effect of allyl isothiocyanate and other mustard oils has not yet been explained. As in a previous experiment (Langer and Štole 1964c), the serum thiocyanate level rose after allyl isothiocyanate. It is also highly probable that thiourea and thiohydantoin derivatives are formed from natural isothiocyanates and amino acids in the gastrointestinal tract or during the culinary preparation of vegetables (Langer, Drobnica and Augustin 1964). These substances have antithyroid effects and probably act like other thiourea derivatives on the initial phases of hormone biosynthesis.

The antithyroid substances present in cabbage and other plants can thus attack biosynthesis of the thyroid hormones in different places. It can be assumed that if key biosynthetic processes are attacked in this manner in several places at once, only a relatively small amount of the individual substances will be needed to cause severe disturbances.

Summary

LANGER, P. (Inst. of Endocrinol., Slovak Acad. Sci., Bratislava.) *Study of Chemical Representatives of the Goitrogenic Activity of Raw Cabbage.* Physiol. bohemoslov. 13(6) : 542–549, 1964.

The author studied several groups of rats fed on a low iodine diet and vegetables (cabbage or carrots) for 20 and 50 days. The diet, vegetable and iodine intake were the same in all the groups.

The control group showed thyroid hypertrophy and other changes in thyroid function resulting from iodine deficiency. Thyroid changes in the group fed on cabbage and a low iodine diet were far severer than in the control group; in particular, the thyroid itself was much heavier. The thiocyanate, total mustard oils and L-5-vinyl-2-thiooxazolidone concentration was determined in the cabbage consumed by this group. Other groups were given different doses of these substances in the pure form and their effect was compared with that of cabbage. A double dose of thiocyanate had about half the effect of cabbage. A dose of allyl isothiocyanate 10–20 times larger than the total mustard oils intake in cabbage had a goitrogenic effect, but a 2–3 times larger dose was ineffective. A dose of L-5,5'-dimethyl-2-thiooxazolidone double the L-5-vinyl-2-thiooxazolidone intake in cabbage had no demonstrable effect on the thyroid.

A mixture of thiocyanate, allyl isothiocyanate and L-5,5'-dimethyl-2-thiooxazolidone in the same doses as in cabbage (in which total mustard oils, the main one of which was allyl isothiocyanate, and L-5-vinyl-2-thiooxazolidone were determined) had a very similar effect to cabbage on the thyroid. The significance of this finding is discussed in relation to knowledge of the chemical factors responsible for the goitrogenic effect of cabbage and other plants.

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ЛАНГЕР, П. (Ин-т эндо[illegible] *остатей структурной а[illegible]* -549, 1964.

Исследовали функции [illegible] рационом с низким соде[illegible] продолжении 20 и 50 дней. [illegible] группы животных.

В контрольной группе [illegible] ее функции в результате [illegible] рационом с низким содержа[illegible] щитовидной железы, чем [illegible] ной железы. В кудрявой [illegible] держание тиоцианата, об[illegible] ными группами крыс полан[illegible] ах действие с действием [illegible] аратов общих горчичных [illegible] в дозе же примерно в 2—3 [illegible] 2-тиоксазолидона в дозе, [illegible] в кудрявой капусте, дости[illegible]

Смесь тиоцианата, аллил[illegible] ах, в каких они принимал[illegible] масла, среди которых прео[illegible] бладала таким действием [illegible] кудрявой капусты. Обсужд[illegible] ных носителей струмигенн[illegible]

Dr. P. LANGER, Endokrin[illegible]

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Резюме

ЛАНГЕР, П. (Ин-т эндокринологии Словацкой АН, Братислава). *Изучение химических основ струмигенной активности сырой кудрявой капусты.* Physiol. bohemoslov. 13(6) : 542–549, 1964.

Исследовали функцию щитовидной железы у нескольких групп крыс, искармливаемых рационом с низким содержанием йода и овощами (кудрявой капустой или морковью) на протяжении 20 и 50 дней. Прием рациона и овощей, равно как и йода, был одинаковым у всех групп животных.

В контрольной группе обнаружили увеличенную щитовидную железу и другие изменения дефицита в результате недостатка йода. Группа, искармливаемая кудрявой капустой и рационом с низким содержанием йода, отличалась гораздо более выраженными изменениями щитовидной железы, чем контрольная группа, в частности, гораздо большим весом щитовидной железы. В кудрявой капусте, потребляемой данной группой животных, определили содержание тиоцианата, общих горчичных масел и L-5-винил-2-тиооксазолидона. Дальнейшим группам крыс подавали различные дозы этих веществ в чистой форме и сравнивали их действие с действием кудрявой капусты. Тиоцианат в дозе, которая в 2 раза превышала прием общих горчичных масел в кудрявой капусте, отличался струмигенным эффектом, в дозе же примерно в 2—3 раза более высокой таким эффектом не обладал. У L-5,5'-диметил-2-тиооксазолидона в дозе, которая в 2 раза превышала прием L-5-винил-2-тиооксазолидона в кудрявой капусте, достоверное влияние на щитовидную железу отсутствовало.

Смесь тиоцианата, аллилизотиоцианата и L-5,5'-диметил-2-тиооксазолидона в таких же дозах, в каких они принимаются с кудрявой капустой (в которой определили общие горчичные масла, среди которых преобладал аллилизотиоцианат, а также L-5-винил-2-тиооксазолидон), обладала таким действием на щитовидную железу, которое очень приближалось к эффекту кудрявой капусты. Обсуждается значение приведенных данных в связи с изучением химических носителей струмигенного действия кудрявой капусты и других растений.

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SYNERGIC EFFECT OF NATURALLY OCCURRING GOITROGENS ON THE THYROID GLAND IN RATS

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In a previous paper it was reported that the goitrogenic activity of cabbage c only partly be explained by the presence of thiocyanate (Langer and Štolc 1964 While studying further goitrogenic factors, several natural mustard oils, such allylisothiocyanate (Langer and Štolc 1965) and also methyl- and butylisothiocy nate (Langer and Greer, to be published) were found to be active.

In subsequent studies, the presence of L-5-vinyl-2-thiooxazolidone, origin found in seeds of Brassica plants only (Astwood, Greer and Ettlinger 1949), b recently also discovered in the edible parts (Kreula and Kiesvaara 1959, Altamur Long and Hasselstrom 1959), was also taken into consideration. Since it is practic impossible to obtain this substance in the pure state, we used a structurally clos similar naturally occurring L-5,5'-dimethyl-2-thiooxazolidone instead. It was show that changes in the thyroid gland in a group of rats fed cabbage are very similar those observed in another group of rats which was force fed with a mixture of thi cyanate, isothiocyanate and thiooxazolidone in doses equivalent to those consum by rats in their daily cabbage ration (Langer 1964). Consequently, we decided make a more detailed study of the synergism of these substances.

Methods

Animals and experimental procedure. 260 male rats of the Holtzman strain were used. The in body weight was 140–160 g. Animals were divided into 9 groups (group 1 consisted of 60 anim the others of 21–34 animals) and each group was further subdivided into 4–6 subgroups (4–6 r each).

Rats received daily different doses of thiocyanate (SCN^-), allylisothiocyanate (AITC) and goit or L-5-vinyl-2-thiooxazolidone (G) for 20 days, in 3 ml of distilled water by a stomach t (Table 1). The control group was given 3 ml of distilled water only. The last dose of antithyr substances was administered on the 21st day and one hour later carrier free I^{131} was inje intraperitoneally (part of group 1 and all rats of groups 6–9 received 10 μC I^{131}, the others 0.2 I^{131}).

Four hours after the administration of radioiodine, blood was drawn from the abdom aorta under ether anaesthesia, the thyroid glands were excised, weighed and their radioactiv measured in a well scintillation counter.

Paper chromatography of thyroid gland hydrolysates. In some rats of group 1 and in all rat groups 6–9 the individual iodinated aminoacids were determined in hydrolysates of the thyr gland using paper chromatography. Thyroid glands were homogenized in 0.5 ml of 0.2% meta merkaptoimidazole (Tapazol, Lilly), then 0.5 ml of 2% pancreatine (Viocase) was added. Be solutions were prepared in a phosphate buffer (pH 8.5). The final pH of the homogenates w

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A scheme of

Group	No. of rats
1	60
2	23
3	34
4	26
5	29
6	22
7	23
8	21
9	22

controlled prior t
was added and h
of the digest wer
acetic acid: water
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the chromatogra
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Preparation of
made, containing
Pure pregoitri
was used for the
pH 7.0). After t
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reaction mixture
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UV-light absorb
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it was extracted
about 90% of G
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Table 1

A scheme of individual groups of rats and doses of antithyroid substances administered

Group	No. of rats	Dose of SCN^- administered to individual rats in mg						Added dose of ATTC in mg		Added dose of G in μg	
1	60	0	1	2	4	8	16	—	—	—	—
2	23	0	1	2	4	8	16	0,5	—	—	—
3	34	0	1	2	4	8	16	—	5,0	—	—
4	26	0	1	2	4	8	16	—	—	50	—
5	29	0	1	2	4	8	16	—	—	—	500
6	22	—	—	2	4	8	16	0,5	—	50	—
7	23	—	—	2	4	8	16	—	5,0	50	—
8	21	—	—	2	4	8	16	0,5	—	—	500
9	22	—	—	2	4	8	16	—	5,0	—	500

controlled prior to hydrolysis and adjusted to the above value, if necessary; then a drop of toluene was added and hydrolysis was allowed to proceed for 18 hours in a water bath at 37°C. Aliquots of the digest were applied to Whatman paper No. 3 and chromatographed in the system butanol: acetic acid: water (4 : 1 : 5). Chromatograms were scanned with an automatic device and the localization of individual peaks was compared with the localization of authentic substances, applied to the chromatogram and detected with the aid of diazotized sulfanilic acid. The area of individual peaks was measured planimetrically and from this the percentage representation of individual substances was calculated, the sum of all areas being taken as 100%.

Preparation of antithyroid substances. A series of potassium thiocyanate solutions (KSCN) was made, containing 1, 2, 4, 8 and 16 mg SCN^- in 3 ml of distilled water.

Pure progoitrin, isolated from Brassica seeds (Brassica napobrassica) according to Greer (1962), was used for the preparation of goitrin. Progoitrin was dissolved in excess 0.2 M phosphate buffer (pH 7.0). After that small amounts of crystalline ascorbic acid and a predetermined volume of a solution of myrosinase prepared by the method of Neuberg and Wagner (1926) were added. After adjusting the final pH to 7.0–7.5, progoitrin was thus hydrolysed for 16–24 hours at 37°C. The rate of hydrolysis was followed at various time intervals by applying aliquot volumes of the reaction mixture on chromatographic paper, which was developed in a water- saturated chloroform system. Goitrin was detected using a low-pressure mercury discharge tube Mineralite as an UV-light absorbing spot, eluted with ethanol and measured spectrophotometrically in the eluate (maximum absorption in ethanol at 243 mμ). When the maximum amount of goitrin was found, it was extracted from the mixture into ether. Ether was evaporated and the residuum, containing about 90% of G (assessment of its purity was done chromatographically as described above), was dissolved in a small volume of distilled water. In aliquots of this solution G was measured spectrophotometrically (on the basis of maximal absorption in water at 240 mμ) and then added to the solutions of SCN^- mentioned above, so that the final concentration was 50 and 500 μg of G in 3 ml of solution. As SCN^- interferes in the spectrophotometric determination of G, reference solutions of G in distilled water were prepared and the content of G was controlled daily during the experiment. No decrease in G content was noted.

Adequate amounts of allylisothiocyanate (Eastmann) were added daily to aliquot volumes of the solutions of SCN^- or SCN^- plus G to reach concentration of 0.5 and 5.0 mg per 3 ml of these solutions immediately before force feeding the animals.

Statistical methods. Individual subgroups receiving the same dose of SCN^- were compared by Student's t-test. The general existence of a statistical difference between groups was complexly checked by combining the resulting probabilities of the individual t-tests by means of the χ^2 distribution with two degrees of freedom per Student's t-test. The repression lines for the weight of thyroid glands were calculated by the method of least squares (Fig. 1), similarly as for the uptake of radioactive iodine; however, in the latter case the original values were first transformed into logarithms and the final results were plotted on the linear scale (Fig. 2).

Results

1. *Body weight*. Final body weight in all groups was within the range 180–220 g and there were no significant differences between the individual groups.

2. *Weight of the thyroid (Fig. 1)*. The weight of thyroid gland increased significantly following the administration of thiocyanate in increasing doses (group 1).

After adding AITC (group 2 and 3) and G (group 4 and 5) to the basic doses of SCN^-, the thyroid weight was greater than after SCN^- administration only (group 1). This difference was highly significant in the majority of cases. In some cases (group 2

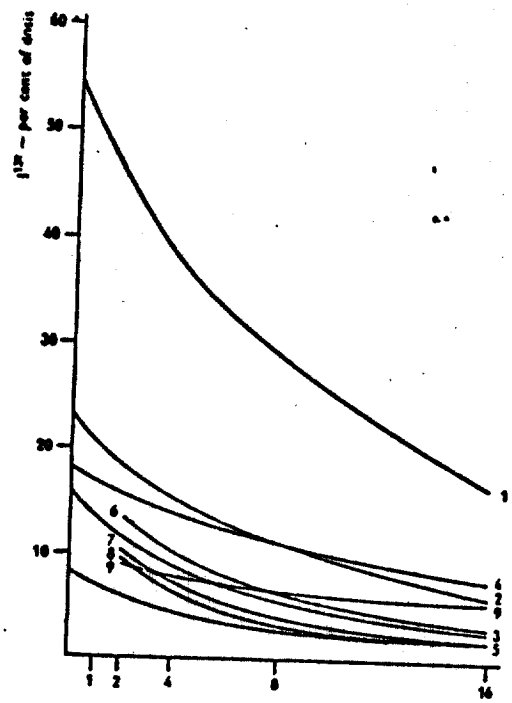

Fig. 1. Weight of the thyroid gland in individual groups (regression curves calculated by the method of least squares). Abscissa: mg SCN^-/day.

and 9), however, the significant difference was found mainly in the range of lower doses of SCN^-. It is necessary to bear in mind, however, that the actual shape of the curves, plotted from the originally measured values differs at some points from the calculated regression curves, given in Fig. 1.

The effect of higher doses of AITC (group 3) was significantly greater than that of lower doses (group 2) and, similarly, higher doses of G (group 5) were more effective than lower doses (group 4). Both doses of G (group 4 and 5) were more effective than either of the AITC doses (group 2 and 3). Since G was administered in doses lower by an order of magnitude than those of AITC, it can be concluded that the former is considerably more potent than the latter.

A higher dose of AITC (group 7) had a significantly greater effect than a lower dose with the same dose of G (group 6). This could not be confirmed when groups 8 and 9 were compared, both with higher doses of G, where a higher AITC dose

group 9) caused
8). If we exclude
in group 9, one h
antithyroid substa
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weight of the thy
AITC and G than
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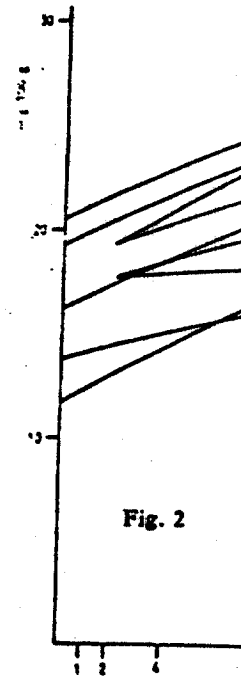


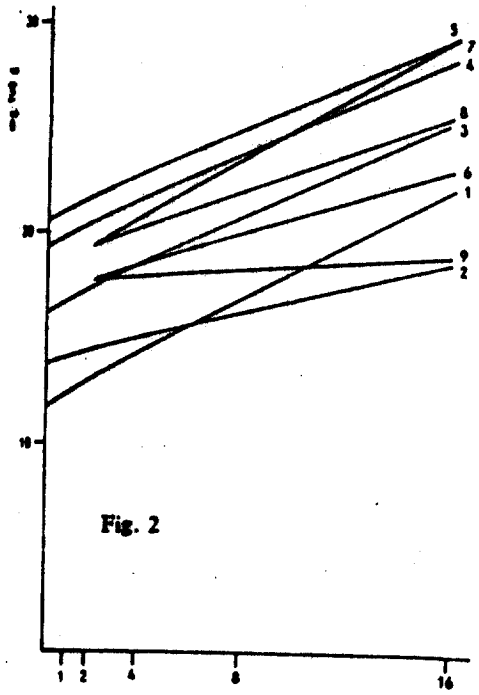
Fig. 2

Fig. 2. Uptake of rad
curves calculated from

Fig. 3. The MIT/DIT
groups (— group 5
of AITC). Ratio MIT
chromatogram. The pe
stances in the chromato

3. *I^{131} uptake af*
doses, the uptake of
other groups the up
Higher doses of A
A higher dose of G
group 4).

(group 9) caused a smaller weight increase of the thyroid than a smaller dose (group 8). If we exclude the presence of an unknown factor, which could modify the results in group 9, one has to concede the possibility that the relation between the dose of antithyroid substances and the response of the thyroid does not apply to this group, which had received the highest doses of goitrogens. And finally, the increase in weight of the thyroid was significantly smaller in group 6 receiving loser doses of AITC and G than in group 7 and 8, which were administered a higher dose of either one of these substances.



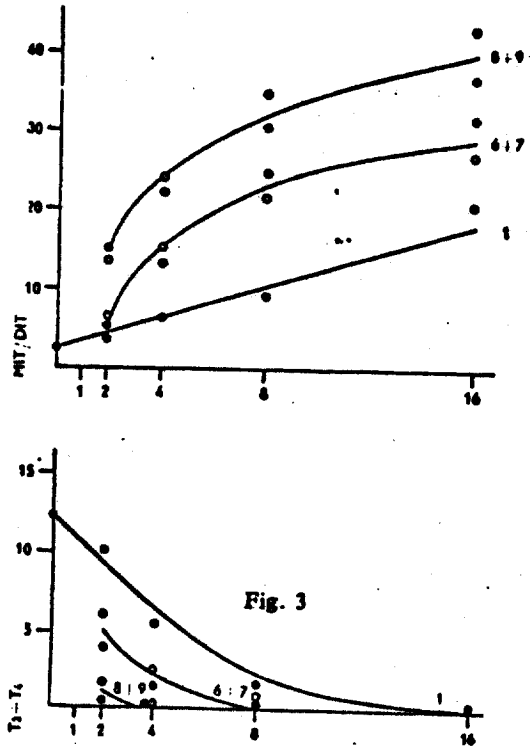


Fig. 2. Uptake of radioiodine 4 hours after the administration in individual groups (regression curves calculated from logarithms of individual values and transformed into linear scale). Abscissa: mg SCN⁻/day.

Fig. 3. The MIT/DIT ratio and the percentage of iodinated thronines in the thyroid gland of some groups (— - group 5 and 8 with lower dose of AITC, — · - group 7 and 9 with a higher dose of AITC). Ratio MIT/DIT was calculated from percentage content of these substances in the chromatogram. The percentage of iodated thyronines indicates the representation of these substances in the chromatograms 4 hours after administration of radioiodine. Abscissa: mg SCN⁻/day.

3. *I^{131} uptake after four hours* (*Fig. 2*). Following a gradual increase of SCN^- doses, the uptake of I^{131} in the thyroid was significantly decreased (group 1). In all other groups the uptake was significantly lower than in group 1.

Higher doses of AITC (group 3) were more effective than lower ones (group 2). A higher dose of G (group 5) also exhibited a greater effect than the lower dose (group 4).

The smaller and larger doses of AITC and G administered simultaneously (group 6-9) were in general more effective than each substance administered separately, while in group 9 no dose-response relationship was again observed.

4. *Biosynthesis of thyroid hormones* (*Fig. 3*). Firstly the ratio of monojodotyrosine to diiodotyrosine (MIT/DIT) was calculated and found to be increased proportionally to increasing doses of SCN^- (group 1). Following higher doses of G (group 8 and 9) the MIT/DIT ratio was significantly higher as compared with that following SCN^- and lower doses of G. There was no clearcut difference to be found between low and high doses of AITC. After lower doses of G (group 6 and 7) values obtained were also significantly higher than after SCN^- only.

Changes in the content of iodated thyronines were also followed (Fig. 3); the percentage representation gradually decreased in the thyroid digests after SCN^- administration (group 1). There was a significantly greater reduction after the low G dose (group 6 and 7) and an even greater one after the higher dose (group 8 and 9), while values in all cases after higher AITC doses were smaller than after the administration of lower doses.

Discussion

In the present paper, three typical representatives of naturally occurring goitrogens in plants of the Brassica family were studied. From the point of view of their antithyroid effect, there are certain differences between them. For example, thiocyanate interferes with the trapping of iodide in the thyroid gland (for review see Wolff 1965) and it might also affect further steps in the biogenesis of thyroid hormones, as its sulphur is oxidized to sulphate in the thyroid (Wood and Williams 1949, Maloof and Soodak 1959). The effect of goitrin is similar to that of compounds of the thionamide type (Iino 1961). The mechanism of action of isothiocyanates, especially that allylisothiocyanate, still remains obscure. We have found its transformation into thiocyanate in rats (Langer 1964) and a thiocyanate-like effect on the release of radioiodide from the thyroid, blocked by tapazol (Langer and Greer, to be published). On the other hand, it has been shown *in vitro* that allylisothiocyanate has a double effect, since it inhibits hormone biogenesis and at the same time reduces the iodide concentration in the thyroid gland.

It was observed in a majority of cases that the effect of two of the above substances on the thyroid was greater than that of one, and furthermore that the net effect of three substances was greater than that of two. The basic assumption was thus proved, namely the existence of synergism between the effect of these substances which interferes with several important steps of thyroid hormones biogenesis. After the termination of the present results, Alexander and Wolff (1965) published a report concerning the synergic action of thiocyanate and synthetic goitrogens of the thionamide type.

The experimental model used does not make it possible to analyse the nature of this synergism biomathematically, namely, whether it is a matter of simple summation or addition of individual effects or whether the action of several substances administered simultaneously is somewhat higher than corresponds to the sum of effects of individually administered substances.

The synergism of naturally occurring goitrogens investigated confirms the assumption concerning the possible explanation of the goitrogenic effects of cabbage and a number of other plants belonging to the Brassica family on the basis of the mechanism, as has been suggested in a previous paper (Langer 1964).

Summary

LANGER, P. (Inst. Endocrinol., Slovak Acad. Sci., Bratislava, Czechoslovakia). *Synergic Effect of Naturally Occurring Goitrogens on the Thyroid Gland in Rats.* Physiol. bohemoslov. 15 (2) : 162-168, 1966.

260 rats were divided into 9 groups. Each group was further subdivided into 4–6 subgroups of 4-6 rats each. Three natural goitrogens were administered to these animals, namely thiocyanate (SCN) in doses 1, 2, 4, 8 and 16 mg daily, allylisothiocyanate (AITC) in doses 0.5 and 5.0 mg daily and goitrin (G) or L-5-vinyl-2-thiooxazolidone in doses 50 and 500 μg daily for 20 days. These substances were administered in various combinations and their effect upon the weight of the thyroid gland, uptake of radioiodine 4 hours after its administration and in some groups on the biosynthesis of thyroidal hormones using paper chromatography was studied.

The more substances were administered simultaneously and the larger their doses, the greater was the antithyroidal effect obtained. It can be concluded from this that the above substances mutually potentiate each other's action. The possible explanation of the goitrogenic effect of plants of the Brassica family on the basis of the simultaneous action of these substances is discussed.

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SOME CHARACTERISTICS OF GASTRIC SECRETION INDUCED BY MUSTARD OIL SUSPENSION[1,2]

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From the Gastroenterology Research Laboratory, The Mount Sinai Hospital, New York City

Received for publication March 10, 1947

In a recent communication from this Laboratory (6) our observations on the consistency, opacity, color, and columnar cell content of gastric mucous secretion were reported. The material was obtained from Pavlov or Heidenhain pouch dogs in response to a number of topical stimuli. The list of agents included 1, gentle massage of the mucosa with a soft rubber catheter; 2, distilled water saturated with ether; 3, five per cent emulsion of clove oil in water; 4, fifty per cent ethyl alcohol; 5, isotonic NaCl (0.17 N); 6, hypertonic NaCl (0.5 N); 7, distilled water, and 8, no experimental stimulus (for control). With any one stimulus, some of the specimens were fluid whereas others were jelly-like; some were transparent or translucent, others were opaque; some contained few or no intact desquamated columnar cells as revealed by microscopic examination of mucous smears, whereas others contained many such cells. The response to the various stimuli differed only in respect to the percentage frequency with which each member of any pair of these characteristics occurred.

One of the numerous stimuli which have been employed by other workers for the investigation of mucous secretion in the digestive tract is 1 per cent aqueous mustard oil (allyl isothiocyanate) (1, 2, 3). This substance has generally been considered to be an acceptable agent for such studies. For this reason it was included in our original investigation, but the response to this irritant differed so radically from the others that it merited wholly separate consideration.

PROCEDURE. The experimental technique was essentially that employed for the other stimuli, and was previously reported in detail (6). The stimulating agent consisted of a 1 per cent suspension of mustard oil (allyl isothiocyanate) in water. In some of the experiments, this was prepared with the wetting agent Tergitol-Penetrant-4 to aid emulsification.[3] The volume of stimulus injected depended upon the capacity of the pouch; in no case was this less than 40 or more than 60 ml. Because of the great irritant action of mustard oil, the total dura-

[1] Preliminary reports of this work were presented before the American Society of Biological Chemists in 1941 (5) and the American Gastroenterological Association in 1944 (4).

[2] This investigation was supported in part by grants from the Anna Fuller Fund, Wyeth Inc., and the United Hospital Fund of N. Y.

[3] A concentration of $\frac{1}{4}$ per cent Tergitol-4 was chosen, not only because it produced effective emulsification, but also because there was no mucus-stimulating action in control experiments using only this detergent at the above concentration. The secretion obtained after its administration resembled spontaneous secretion in secretory rate and appearance. However, control experiments with $\frac{1}{2}$ per cent Tergitol-4 gave a rate of stimulation slightly greater than that for spontaneous secretion. Tergitol-Penetrant-4 was kindly supplied by its manufacturer, Carbide and Carbon Chemicals Corp., New York City.

tion of stimulation was usually only 5 minutes. In a few instances when it was 10 minutes, the total period was divided into two consecutive 5-minute intervals by the aspiration and reinjection of the material. This was done in order to refresh those portions which were in direct contact with the mucosa. Since the secretory response following a treatment continued for more than 5 hours, instead of 2–3 hours as with the other stimuli, the present experiments were stopped before the rate had fallen to its control (pre-stimulation) value. The duration of the experiments was not uniform, because of other objectives. The time interval for the collection of single samples also varied according to the requirements of the individual experiment. In all, 15 experiments were performed with mustard oil, using 7 Pavlov or Heidenhain pouch dogs. All of these animals had been employed for the experiments using the other agents. However, the mustard oil experiments were not performed in succession but were intermingled with the others.

RESULTS. *Duration and rate of secretion.* After application of the stimulus, about 10 minutes were required to set the dog up in the collecting stand and to drain most of the stimulus out of the pouch. During this combined period of stimulation and drainage (about 15 min.) some secretion undoubtedly took place. Hence it is impossible to determine a "latent period" between stimulation and beginning of secretion. Following drainage of the stimulating agent, secretion was collected for 5–15 minutes; this was designated the "initial specimen" of the experiment. The variations in secretory rate for subsequent collection intervals are illustrated in figure 1, which depicts the results of one of our best experiments. In general the duration of flow following a single application of the stimulating agent was 5 hours or more. The rate of secretion during the initial collection period was higher than during any subsequent one in all but 4 of the 15 experiments. Immediately following this initial high value, the rate began to fall off, the change being rapid at first and then progressively slower. The curvilinear trend depicted by the graph in figure 1 is fairly typical of the entire series of experiments. By replotting these data on semi-logarithmic paper (fig. 2) its exponential character becomes evident. In only 3 of the 15 experiments was the trend curve strikingly different more or less flat and in all of these cases the over-all rate of secretion was so low that they can be characterized as atypical.

For the 15 experiments included in the present series the highest initial rate of secretion was 17.1 ml. per 15 minutes; the mean of these 15 values was 11.1, with a standard deviation ($S.D._M$) of 1.34 ml. A total of 107 specimens were collected in these experiments; the highest rate of secretion was 19.8 ml. per 15 minutes and the mean was 5.26 ($S.D._M$ = 0.45). All average values were calculated without weighting for volume or time-interval. An analysis of these several data, according to whether the mustard oil suspensions were prepared with or without Tergitol, revealed no significant difference which might be ascribed to the use of this wetting agent.

Consistency of the secretion. Specimens obtained in the experiments with mustard oil were generally fluid enough to drip continuously from the collecting catheter. Of 46 specimens collected with mustard oil in the absence of Tergitol,

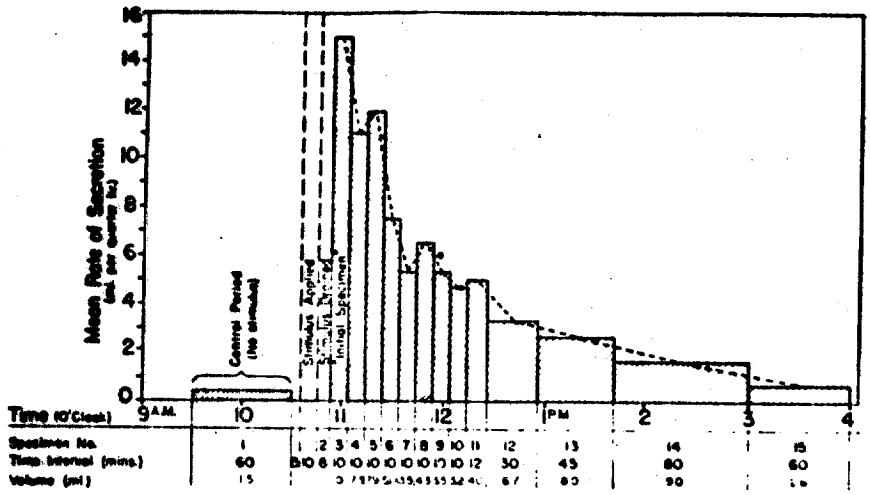

Fig. 1. Secretory response of dog's Heidenhain pouch to 1 per cent aqueous mustard oil suspension (expt. Mu.-278).

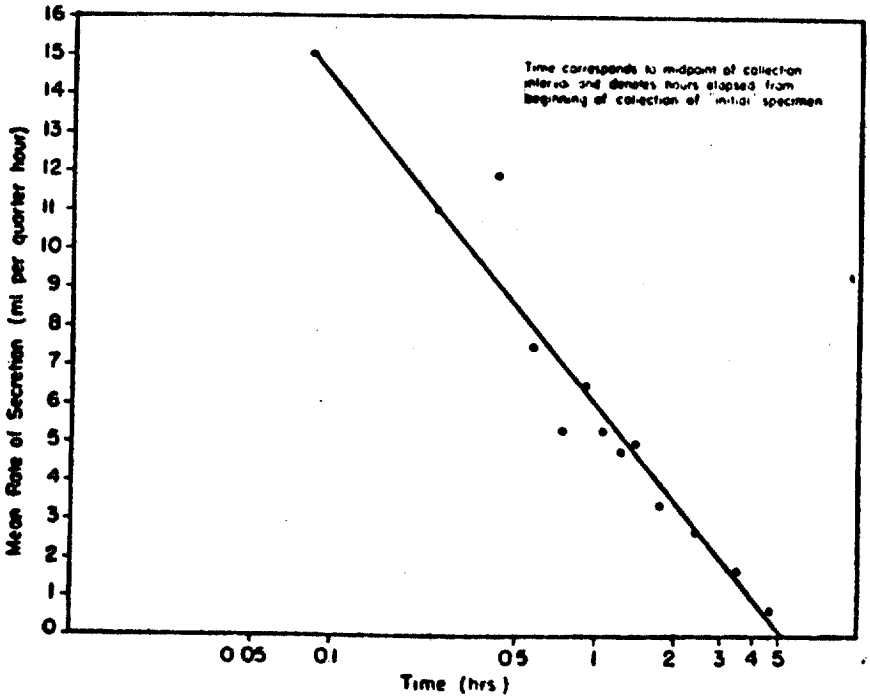

Fig. 2. Secretory rate data plotted against logarithm of time (expt. Mu.-278).

1 sample (2 per cent) was characterized as viscous or jelly-like. For the specimens obtained with the addition of Tergitol, the results were only slightly different; of a total of 61 specimens, only 6 (10 per cent) were viscous. The

difference between these two percentages is not significant statistically ($P = 0.10$) but they are both considerably smaller than the control value of 45.7 per cent derived from spontaneous secretion.

In contrast with the fluid specimens obtained in response to the previous stimuli, those induced by mustard oil gelled on standing at room temperature. Sometimes this process started during the collection period; at other times it was observed 5 to 30 minutes thereafter. Usually the clot seemed to include the entire volume of specimen; on standing overnight in the refrigerator, however, it retracted and a supernatant layer of fluid was noted. In appearance and texture, this coagulum differed strikingly from jelly-like mucus in that it was translucent and much softer. Although it cohered on pouring, the mass was easily broken up and a uniform mixture created by vigorous shaking.

Color. The fluid obtained directly after stimulation with mustard oil had the amber appearance of blood plasma. Some time before the third hour following removal of the stimulus, the color became faint pink; in subsequent specimens, this continued to deepen to a decided red. The sanguinous origin of this color was confirmed both chemically (by guaiac test) and microscopically (by the presence of erythrocytes). Tergitol exercised no appreciable influence on this sequence of colors.

Opacity. Although experiments with and without Tergitol manifested no difference in regard to bleeding, they did with regard to opacity of the specimens. In the absence of this emulsifier, the specimens were almost always transparent or translucent; only 2 in a group of 39 (5 per cent) could be described as opaque. When Tergitol was used, however, the proportion rose to 23 in 61 (38 per cent) a difference which is clearly significant. In both groups of experiments, the typically clear specimens sometimes contained small opaque particles in suspension. However, without Tergitol the frequency of occurrence of such particles, and their density per specimen, were characteristically low—frequently near zero—whereas when mustard oil-Tergitol was used, both frequency and density were high. Control experiments with Tergitol alone in a concentration of $\frac{1}{10}$ per cent gave rise to secretion resembling, both in rate and in appearance, that obtained spontaneously.

Presence of columnar cells. Systematic examination of mucous smears was performed in 6 of the 7 experiments without Tergitol, and yielded 31 specimens for which both opacity and cell content were recorded. All but one of these were non-opaque; all of them were found to contain very few or no columnar cells on examination of representative smears. However when smears were deliberately prepared to include one or two of the opaque particles referred to in the previous paragraph, many cells were noted. Microscopic examination of these particles after staining revealed them to be bits of surface epithelium, such as were obtained with some of the other stimuli and described in the previous report. Those particles were surrounded by many individual columnar cells—particularly dense at the periphery of the particle, less so at a slight distance.

Discussion. Mustard oil (allyl isothiocyanate) in aqueous suspension has been used by several investigators in studies on mucous secretion in the digestive tracts of experimental animals. From such reports, however, it is apparent that

this agent induces an inflammatory reaction as well as a secretory response of the mucous epithelium. In our own experience, the material obtained from pouches stimulated with mustard oil differs markedly from that secreted after application of other agents employed as mucus stimuli. This can be demonstrated by a comparison of the data obtained in the present investigation with those previously reported from this Laboratory (6) for spontaneous secretion, mechanical stimulation, and contact with aqueous solutions and suspensions having various irritant potencies.

Concerning rate of secretion, none of the 425 specimens obtained with the stimuli used previously ever yielded values higher than 2.3 ml. per quarter hour, and the majority of them were well below 1.0, whereas the highest value for mustard oil secretion was 19.8 ml. and the mean was 5.3. Furthermore, the entire duration of the former experiments—i.e., the time required for the secretory rate to return to its control value, the rate of spontaneous secretion—rarely exceeded three hours. In contrast with this, the mustard oil experiments lasted at least twice as long, and even after this extended interval, the rate had not yet fallen to the control level. No comparison of the two sets of experiments is possible in regard to the shape of the secretory-time curves, because of the quantitative inadequacy of the rate data of the experiments with the other stimuli. As previously explained, this arose from the gelatinous character of a majority of these specimens. Hence, judging by time and volume relations alone, it might be inferred that the mustard oil suspension is a far more potent stimulus than any which we have previously investigated. Other characteristics of the response discussed in the following paragraphs, however, suggest a radically different conclusion.

With only rare exception, the consistency of the specimens of secretion collected in the mustard oil experiments was recorded as fluid. Regardless of the presence or absence of Tergitol, only 6.5 per cent were viscous or jelly-like, as compared with 45.7 per cent previously reported for the specimens of "spontaneous secretion". In fact, the former value is lower even than that obtained after application of *isotonic saline* solution (13.3 per cent), but the difference between the two has no statistical significance. Our earlier work led to the conclusion that the incidence of mucus of high viscosity is probably correlated directly with the irritant power of the stimulus. In contrast with this, we find that a 1 per cent suspension of allyl isothiocyanate—which may be more irritating than most or even all of these other stimuli—yields only a negligible incidence of jelly-like specimens, and therefore might be classified as a mucus stimulus of very slight potency.

In regard to opacity, it was found that the mustard oil suspension prepared with Tergitol gives a higher incidence of opaque specimens than does the Tergitol-free suspension—38 per cent as compared with 5 per cent. The influence of the detergent will be discussed later. However, even the former value is considerably less than the values obtained with 50 per cent ethyl alcohol, hypertonic NaCl solution, water saturated with ether, and 5 per cent clove oil suspension in water—which vary between 82 and 96 per cent. Furthermore, the over-all

incidence of opaque specimens in all mustard oil experiments (with and without Tergitol) is almost identical with that for spontaneously secreted mucus—25 per cent and 27 per cent respectively.

Unfortunately, only one of the experiments with Tergitol-mustard oil included microscopic examination for columnar cell content. In the 6 experiments without Tergitol, however, the incidence of specimens with "many" columnar cells was only about 3 per cent, and the correlation of high (and low) cell content with opacity (and non-opacity) was extremely high. This percentage value is of the same order of magnitude as that obtained with isotonic saline (7 per cent), and considerably less than that for spontaneous secretion (30 per cent). However, small isolated areas of high columnar cell density were sometimes found, even in slides with an otherwise low density, but this localized occurrence of individual cells probably results from an occasional single clump of epithelial cells rather than from a diffuse, generalized desquamation. It appears, therefore, that the mustard oil suspension without Tergitol does not induce the extensive desquamation characteristic of other stimuli, and though the addition of Tergitol does cause desquamation to some degree, it is not nearly as extensive as with the other stimuli. It is not unlikely that the clumps of surface epithelium which occur occasionally in the mustard oil specimens are shed "spontaneously", since such clumps are seen with about the same frequency as in the specimens of "spontaneous" secretion. From this it seems evident that the mustard oil induces little or no loosening of the intercellular cement substance.

Concerning the influence of Tergitol-4 on the action of the allyl isothiocyanate, it is clear that there is none so far as the time relation of fluid output, the extent of bleeding, or even the consistency of the secretion are concerned. However, in regard to the opacity a difference exists between the experiments with and without the wetting agent, specimens from the former being slightly, but significantly, more opaque than those from the latter. Although the data on the microscopic appearance of the Tergitol-mustard oil specimens is deficient, there is evidence to indicate that they contain a slightly greater number of clumps of columnar cells than do the others. A similar correlation between opacity and incidence of epithelial cells was demonstrated in our report with the other stimuli as well. It is difficult to see why the presence of Tergitol should result in this slight increase in cell content of the specimens, particularly since control experiments with the detergent alone yielded material which differed in no way from that previously described as "spontaneous secretion". It may be that the detergent loosens portions of mucus adherent to the mucosa in the resting state, together with occasional bits of epithelium previously desquamated and enmeshed in this mucus. On the other hand, the peculiar response to the mixture of mustard oil and Tergitol may result from the potentiation of one by the other, either by actual chemical combination or because of the surface activity of the wetting agent.

Thus, in terms of consistency, opacity, and columnar cell content, a 1 per cent emulsion of mustard oil may be deemed to be no more potent as a mucus stimulus than is isotonic saline. On the other hand, both rate and duration of secretion

suggest an intensity of stimulation by the mustard oil emulsion which exceeds by far that manifested by any other agent which we have so far studied. This is in accordance with the fact that 1 per cent mustard oil would ordinarily be classified by pharmacological criteria as considerably more irritating than any of these other mucus stimuli. It may be thought that the striking difference between the results with mustard oil and those with the other agents arose from a pathological conditon of the mucosa, e.g., a gastritis, which obtained at the time the present experiments were performed but was absent during the experiments previously reported. This is precluded, however, by the fact that the mustard oil experiments were irregularly distributed among all the others in point of time of their performance.

In attempting to resolve this paradox, several factors must be kept in mind. The total volume of secretion obtained in response to a single application of the mustard oil suspension is many times greater than that following application of even the most potent of the stimuli previously employed. Also in both groups of experiments, metachromatically-staining material (presumably mucin) was repeatedly found to be present, but the quantity in the mustard oil specimens appeared to be considerably less than in the others. Unfortunately, at the time these investigations were performed, there was available to us no acceptable analytical method for determining concentration of mucin, so that our comparisons have been made on a qualitative rather than a quantitative basis. In spite of the presence of mucin, however, the mustard oil specimens bear a closer resemblance to blood plasma or transudate than to fluid mucus. This is evidenced by their color—which is amber during the first hours of collection, and pink to red during the latter part—and also by their slow congealing shortly after collection. This gradual yet persistent appearance of blood is another manifestation of an inflammatory reaction induced by the stimulus. There is little likelihood that the bleeding results from traumatization by the collecting apparatus. Such oozing of blood was never observed with the other stimuli, and constitutes further evidence that transudation (or exudation) bears the major responsibility for the lack of concordance between the mustard oil experiments and the others.[4] Thus the paradox can be resolved by the recognition of the fact that we are dealing here with a mixture of transudate (or exudate) and mucous secretion, in which the former is in considerable excess over the latter.

CONCLUSIONS

It may be concluded from the foregoing that a 1 per cent emulsion of mustard oil in water is a very poor stimulus for gastric mucus secretion, in comparison with the agents previously studied. On the contrary, this agent is an active mucosal irritant, giving rise to large amounts of a "secretion" which consists for the most part of serous transudate (or exudate). The persistently low

[4] It is noteworthy that the pH values of mustard oil specimens are generally greater than 7.5, the uppermost limit reported by Menkin (7) for lymph and inflammatory exudate. Many of the values fall between 8.0 and 8.5. The significance of these findings in relation to the present study will be considered in a later report.

viscosity, low order of opacity, low incidence of columnar cells, and probable small mucin content—all indicate that mustard oil in this concentration fails to evoke the usual responses of a true mucus stimulus. On the other hand, the high volume, rate, and duration of secretion; the resemblance in color of early specimens to blood plasma; the almost invariable appearance of fresh blood, increasing in intensity with progress of the experiment—all these, together with the observations on viscosity, opacity, etc. support the view that the "secretion" is a transudate or exudate. Undoubtedly, some mucus is present in the "secretion", but the proportion seems to be very small indeed. It is evident, therefore, that mustard oil emulsion should not be used as a stimulus for investigation of mucous secretion in the stomach.

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Electron microscope studies on the effect of irritants on the epidermis of the mouse

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Summary

The changes in the epidermis of albino mice under the action of mustard oil, turpentine oil and cantharidin are studied as a function of time with the aid of an electron microscope. The irritants used cause deposition of DNS material on the inner nuclear membrane; differences appear in the subsequent development.

Mustard oil: During the first hours after the application, an increase of promitochondria and of vesicles of the endoplasmatic reticulum is observable. Only after several hours there occurs a swelling of the mitochondria, accompanied by intense vacuolization of the cytoplasm. After gradual regression of the lesions there develops after several days a hyperplastic epidermis with typical formation of the individual strata.

Turpentine oil: Destruction of the mitochondria sets in after a short time and persists for days. Vacuolization in the cytoplasm is moderate, the material of the nucleus being very lumpy. Normalization takes much longer, and the pronounced hyperplasia of the epidermis is missing.

Cantharidin: The changes under our test conditions are small. On the mitochondria disorders occur after one to two hours, the other cell structures show hardly any deviations. The regression is fast, after a few days the epidermis is altered hyperplastically.

The results are discussed with respect to the mechanism of action of the irritants used. Special attention is given further to the question as to the nature of the keratohyalin granules.

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Studies on the submicroscopic structure of the epidermis have been carried ou

especially on human skin (for literature cf. (17)); to clarify the organization of the "basal membrane" predominantly amphibia skin was used (literature cf.(32)) Few observations exist on the normal epidermis of mice (23, 33, 38) and of rats (11, 19,33).

Some studies are based on experimentally influenced skin. Albertini (1) deal with carcinoma caused by methyl cholanthrene; Setälä and Stjernvall (34) investi ated the effect of Tween 60; Hoffmann (18) observed the alterations upon infecti with mouse pox; and LeBlanc (22) dealt with exposure to cold. Observations on inflammatory lesions in the epidermis have not become known to us thus far; some papers exist on pathological changes on the mucous membrane of the mouth (9, 36, 37).

The present studies had the purpose to observe by means of the electron micro scope specific alterations in the submicroscopic structure of the epidermis unde the influence of various irritants.

Material and method

The tests were carried out on the skin (ear, belly, back) of white mice of bo sexes at an age of 6 to 9 weeks. The selection of the resting phase proved nece ary because of the varying reaction of the skin as a function of the cycle of hair growth.

Before application of the irritant, the animals had the hair removed with scissors on an area of about 2 cm^2. Chemical depilation alters the epidermis an is therefore not suitable.

As irritants there were used allyl mustard oil, turpentine, and cantharidin. Mustard oil and turpentine were dropped on the skin undiluted, cantharidin was applied as a cantharide extract in alcohol or in the form of cantharoplast (0.2% cantharidin).

Skin samples were taken after different intervals of time (5 minutes to 6 days the skin of untreated animals serving as control. All preparations were examine under optical and electron microscopes. The histological comparison was made wi paraffin sections stained with hematoxylin-eosin, also Feulgen and various gly- cogen stainings were done. For the examinations in the electron microscope the

usual methods were followed. The material was embedded in methacrylic acid est In some cases a pretreatment with ribonuclease or n HCl was applied. The electr microscopic photographs were taken with the electromagnetic SEM2.

Results

The structure of the epidermis is essentially the same in the various region only in the vicinity of the follicle openings differences appear. During the resting phase of the hair cycle it is difficult to differentiate the strata of t epidermis - with the exception of the stratum corneum - while they appear distinctly during the growth phase.

Figure 1 gives a surveyof the structure elements of the epidermis cells. The photograph was taken after short action of mustard oil, but the organization of the structures corresponds in principle to the normal epidermis.

Normal epidermis

The epithelial cells of the epidermis are separated from the cell-poor, fiber rich cutis by a boundary lamella. This epidermis-cutis boundary (fig. 1E) is formed by a double membrane; the lamella toward the epidermis is the cell membrane of the basal cell, while the second lamella is separated from it by a zone about 300 ang wide, fairly pervious to electrons. This adepidermal membrane is approximately 150 ang thick; adjoining it are the connective tissue fibers of th cutis. At some points fibrils seem to be in direct contact with it (fig. 2a).

The cells of the stratum basale contain a usually lengthwise oval nucleus; their plasma appears fine-grained due to numerous free ribonucleoprotein (RNP) granules; only few elements of the endoplasmatic reticulum, frequently in the form of vesicles of the agranular type, are present (fig. 3a). The mitochondria (fig. 4a) lie preferentially in proximity of the nucleus; tonofilaments extend i the periphery of the cell, so that there results a perinuclear filament-free zon The tonofilaments end at the contact zones and never cross the cell boundaries. The contact zones (fig. 1D) have a complicated structure, which in its fundamental features matches the description of Odland (29).

The same structures appear in the stratum spinosum; the tonofilaments, which merge to form tonofibrils, are more defined. Besides, the stratum spinosum show

a stronger folding of the cell membranes, which diverge only occasionally, maki room for narrow intercellulars.

The cells of the stratum granulosum are flattened parallel to the surface an often contain only residues of nuclei; their cell boundaries extend relatively linearly. Characteristic are the rounded, highly electron-dispersing keratohyalin granules (cf. fig. 9). Mitochondria, RNP granules and elements of the endoplasmatic reticulum decrease in number. The tonofibrils pass through the ce in a preferred direction and show a changed appearance because of the keratiniza tion taking place in this cell layer.

The cells of the stratum granulosum generally change into the totally keratinized lamellae of the stratum corneum without transition. These are extremely flattened cells, with fine-granular cell content; at some points these granules make the impression of a fibrillar arrangement in longitudinal direction of the cell (fig. 5a).

Alterations after the action of mustard oil

The epidermis alterations begin 5 minutes after mustard oil application with loosening in the stratum corneum, which after a while leads to dissolution of th horn lamellae (fig. 5b). At that time no change is as yet observable in the oth cell structures. After 15 minutes one sees in the upper portion of the epidermis narrow intercellulars and occasionally small vacuoles proximate to the nucleus. Simultaneously with the vacuolization a transformation of the nuclear material occurs at the cell nuclei, and accumulations of highly electron-dispersing material are evident at the inner nuclear membrane.

In later stages the vacuolization spreads to the entire epidermis. The vacuoles are surrounded by a membrane and occur free in the cytoplasm as relatively small forms, while the large ones are found predominantly in the vicinity of the nuclei (fig. 6). Besides, the intercellulars are greatly enlarged, and large cavities form over the epidermis-cutis boundary. All cavities contain fine-grained material. At that time, in addition to unchanged mitochondria, numerous small mitochondria with only indistinctly discernible internal structures can be observed (fig. 4b); the elements of the endoplasmatic reticulum are developed

more distinctly and are covered with RNP granules (fig. 3b). Also the outer nuclear membrane has numerous RNP particles deposited on it. Sometimes structure are observable which may be regarded as ergastoplasm vesicles (fig. 7x).

After a few hours the epidermis is marked by a massive vacuolization (fig. 8) Sometimes the vacuoles fill almost the entire cell and in part have no membrane delineation, whereas greatly enlarged intercellulars generally do not occur. In this stage, besides the swelling of the endoplasmatic reticulum (fig. 8eR), ther appears also a swelling of the mitochondria, which is accompanied by a destructio of the cristae mitochondriales and leads to the formation of vacuoles in the plasma (fig. 4c, fig. 8M). In addition, subcorneal blisters are observable, whi develop either at the boundary between stratum corneum and stratum granulosum or result by the bursting of the lowest inflated horn lamella.

After 24 hours, the vacuolization has regressed; there are no large vacuoles i the cells. The structures of the endoplasmatic reticulum are swollen only in pa and the number of damaged mitochondria is reduced. In the upper part of the epi ermis only narrow intercellulars appear, while between the basal cells they ofte assume larger dimensions. They differ, however, from the previously formed inte cellulars by the formation of freely ending plasma processes (microvilli) and by the almost complete absence of contact zones. It is into these intercellular spaces that the leukocytes immigrate; this occurrence is accompanied by an enlargement of the adepidermal membrane (fig. 2b) and a local disturbance of the line of the epidermis-cutis boundary. In the cells, strong tonofibrils appear in greater number, the mitochondria are often small with only indistinct interna structures.

In the course of a few days these symptoms regress and there evolves a hyperplastic epidermis, the cells of which are marked moreover by a slight hypertroph (fig. 9). The nuclei are large and show an almost uniform distribution of the nuclear material; usually they contain well marked nucleoli. The epidermis now shows all layers in a typical formation as known from the human skin. Adjacent to the cells of the stratum basale withlarge, well developed mitochondria and an almost rectilinear course of the cell walls is the multi-row stratum spinosum.

Here we have typical "spines", in that the cells are connected via contact zones with plasma processes (fig. 10D). The multi-layer stratum granulosum contains ma keratohyalin granules (fig. 9 Kg), which, like the mitochondria, occur preferenti ally in proximity of the nucleus. By deposition of material on the keratohyalin granules, irregularly formed bodies result. Between the lower-lying cells the cell membranes are convoluted, while in the upper layers they are almost straigh Outwardly a stratum corneum composed of numerous wide and homogeneous horn lamel forms the termination.

Alterations after action of turpentine

After the action of turpentine, too, loosening of the stratum corneum soon appears, leading to similar phenomena as after mustard oil (cf. fig. 5b). First alterations in the mitochrondria are observable already after 5 minutes; after 1 minutes a considerable portion of the mitochondria is marked by disappearance of the matrix and reduction of the cristae mitochondriales, and upon their complete loss finally vacuoles are formed in the cytoplasm (fig. 11). The swelling of th mitochondria, however, is slight. The cell nuclei show deposition of electron-impervious material on the inner nuclear membrane and are at times irregularly formed. No distinct deviations from the normal state appear in the other cell structures.

After 30 minutes the cells are already more severely damaged, in that by loos and inhomogeneous distribution of the free RNP granules membraneless cavities of varying size are formed in the cytoplasm. The nuclear material is distributed i the nuclear space irregularly in lumpy accumulations (fig. 12). In later stages these phenomena increase; the nuclei are often irregularly contoured, they conta only lumpy nuclear material. In part a plasma-poor zone has formed around the nuclei. The mitochondria show - besides individual dense compact forms - loss of the internal structures; they are somewhat swollen, as are also the few elements of the endoplasmatic reticulum. In addition to these vacuolous structures there occur membraneless larger and smaller cavities; occasionally narrow intercellulars are formed. The adepidermal membrane was enlarged here and there (cf. fig. 2b).

After one day we still find many mitochondria with disturbed internal structures, nuclei with lumpy nuclear material, and numerous cavities in the cytoplas In contrast to the alterations after mustard oil action, the reconstitution of t epidermis takes much longer, as can be seen also from the persistent slight vacu ization of the cytoplasm and the perturbation of the mitochondria (fig. 13). Thi is reflected also in the missing hyperplasia after 6 days and the missing typica stratification connected therewith. The number of tonofibrils increases, but th number of keratohyalin granules remains relatively small. However, the increased keratinization suggests that epidermis hyperplasia will follow.

Modifications after action of cantharidin

Blistering was not observed. However, it is known from other studies (15, 30 that animal skin has little ability to blister. Under the above described test conditions, the changes in the epidermis cells are small.

After 30 minutes, all cell structures are normal, apart from an occasional appearance of narrow intercellulars. After one hour, the nuclei become conspicu ous through deposition of electron-impervious material on the inner nuclear membrane; the cells contain strong tonofibrils and numerous free RNP granules. The most important alteration observable is a disturbance of the mitochondria (fig. 14). This phenomenon increases somewhat as time goes on. The occurring mitochondria are essentially small compact forms without distinct cristae mitochondriales. A slight vacuolization is observable in the cytoplasm, cavities with membrane delineation and without occurring simultaneously. The intercellulars are limited. The membrane-positioned material accumulations in the cell nucleus are less pronounced. These alterations regress quickly. After cantharidin action of one day already, the epidermis appears enlarged due to increase of the cell strata, which, however, are not clearly delimited from each other (fig. 16) The numerous keratohyalin granules are not seen in the stratum granulosum, but there appears a number of usually intact mitochondria and numerous vesicles of the endoplasmatic reticulum. The cells of the stratum spinosum and stratum basa contain evenly formed nuclei with very uniformly distributed nuclear material. The numerous mitochondria prefer proximity of the nucleus and contain clarly

recognizable internal structures. There are few tonofilaments (fig. 15).

After several days, a pronounced hyperplasia of the epidermis, as after musta oil application, has developed. The cells in the stratum granulosum are characterized by the spread of mitochondria (M), of the endoplasmatic reticulum (eR), and especially the appearance of horny substances (K) and keratohyalin granules (Kg) as well as numerous tonofilaments (F) (Fig. 17). Because of the minor form tion of intercellulars, the characteristic aspect of the "spiny cells" is missin in the stratum spinosum. Typical of the cells of the stratum basale are especially groups of large mitochondria at the basal nuclear pole.

Discussion

The morphological approach is only one method of exploring the interaction of form and function. It is therefore indispensable to make use of biochemical and other results for interpretation. Since it is known from numerous studies that the action of an irritant leads to modifications of the cellular metabolism, attention is centered on cell structures which are directly linked with metabolism The first changes, both after mustard oil and, in lesser degree, after turpentine oil application, consist in a loosening of the lamellae of the stratum corneum, whereas this effect is absent after the action of cantharidin. Keratolysis tests performed for comparison indicate that this phenomenon is attributable to keratolytic properties of the two substances. The blistering horn lamellae become detached as "ballooned cells", and in part the membranes burst at certain points. Hoffmann (18) describes similar formations on granulosum cells in infectious ectromelia and attributes some importance to them in the propagation of viruses. In our tests we are certainly not dealing with a specific phenomenon.

The mitochondria are the carriers of cell respiration. They do not show degeneration during the first hours after mustard oil application, on the contrary the numerous small mitochondria with indistinct internal structures presumably represent "promitochondria" (40), suggesting a multiplication of the mitochondr

Mölbert (26) describes the same phenomenon in histotoxic hypoxydoses on liver cells and interprets them in the sense of an increase in activity. In agreemen therewith, an initial increase has been observed in metabolism studies on liver

cells (14) at low mustard oil concentrations before the decrease of respiration, while on isolated frog's skin immediate inhibition of respiration occurs under the influence of allyl mustard oil (12). This apparent contradiction with the morphological finding can be explained by the different test conditions, among other things.

Not until several hours after the mustard oil application do the mitochondria begin to swell, with loss of the internal structures. It may be assumed that besides the direct damage to the mitochondria, a breakdown of the osmotic regula tion of the cell is mainly responsible for this, resulting in an influx of exuda tion liquid. The vacuolous modification of the mitochondria as a reaction of th cell has been observed under very different conditions; a similar delayed mito-chondria destruction has been described by Mölbert (26) in histotoxic hypoxydosi Whether later a regeneration of the vacuole-modified mitochondria to intact form occurs (13, 27), it was not possible to decide.

The increase in promitochondria and the epidermis hyperplasia occurring after a few days were observed both after cantharidin application and after the action of heat. The numerous large mitochondria suggest an increased metabolism in the hyperplastic epidermis. This can be inferred also from studies on rat epidermis modified hyperplastically by croton oil (7).

The effect of turpentine on the mitochondria differs from that of mustard oil in that after 5 minutes already a loss of matrix begins, which leads to disappea ance of the cristae mitochondriales and then to vacuoles in the plasma; the mito chondria, however, undergo only slight swelling. This rapid mitochondria modi-fication points to direct attack of turpentine on respiration, as is suggested also by the immediate inhibition in the respiration test (14). As it appears th the promitochondria occurring after several hours are often inhibited in their further development and disturbances of the internal structure of the mitochondr are observable days later, one must assume that the harmful effect of turpentine remains active in the cell quite long.

For cantharidin, our studies showed little damage to the mitochondria, appear ing after about one hour. After about two hours, there is an increase in the pr

mitochondria. In agreement with this, there is seen in metabolism tests after approximately one hour a slight decrease in respiration, soon compensated depen ing on the concentration (14). As cantharidin is regarded as a pure cell poison (16), it is strange that there is not more effect on the structures of the epide mis cell. The mitochondria changes are much less than those observed with turpe tine application. The effect of cantharidin is quicker to appear than that of mustard oil. But while after mustard oil application the swelling of the mito-chondria seems to be primarily a secondary effect, with cantharidin it seems to be a primary effect, despite the late onset of the modification, as with turpen-tine.

Cytoplasm being the seat of most glycolytic enzymes of the cell, changes appe ing in the cytoplasm must also have an influence on the cellular metabolism. Aft mustard oil application, vacuoles appear in the cytoplasm at an early stage. They often contain fine-grained material, presumably corresponding to protein precipitations. The vacuoles situated near the nucleus frequently constitute en largements of the double-walled nuclear membrane and are explained by the partic ular susceptibility to swelling of the "perinuclear space" (39). Vacuolization in the plasma and in proximity of the nucleus occurs under various conditions; i reflects a displacement of the water balance. The reversible elimination of liq in vacuoles represents a high energy demand on the cell (2). According to Thema (37), this vacuolization can be regarded as the expression of a hypertrophic gly colysis. This is in agreement with the initial increase in glycolysis measured in metabolism tests (12, 14). It may be assumed thatin this stage the cells hav not yet suffered severe damage in the energy economy, this being in agreement al with the mitochondria findings discussed.

The progressive vacuolization, which finally leads to absorption of water fro the surroundings and to a complete imbibition of the epidermis with exudate, mus be regarded as the morphological expression of a glycolysis inhibition occurring later. This, according to Bacq (3), involves reactions between SH groups and th mustard oil. The formation of wide intercellular spaces is connected with the capillary permeability increased by mustard oil. Simultaneously with the swelli

of vesicles of the endoplasmatic reticulum and of the mitochondria, the appearance of cavities in the plasma not enclosed by a membrane is observable. These phenomena point to a greater disturbance in the energy metabolism. All cavities and vacuoles contain barely any granular material ; this, too, suggests an osmotically caused influx of water.

In contrast to the effect of mustard oil, turpentine in the epidermis cells brings about immediately more or less large cavities not surrounded by a membra and leads to lumping of the cytoplasm. The small amount of intercellular edema and hence the missing imbibition of the cells with exudate results from the bri action of the turpentine on the capillary resistance.

The alterations in the cytoplasm after cantharidin application are so small that the results must be regarded with some reserve, as the cantharidin was not available in substantial amount. The almost complete absence of intercellular spaces suggests a very slight effect of cantharidin on capillary permeability (16). The later effects of cantharidin seem to be a certain inhibition of the cell differentiation, manifesting itself, among other things, in the delayed keratohyalin granule formation and in the reduced formation of "spiny cells" in the hyperplastic epidermis.

In the epidermis cells, as in all quickly dividing cells, the endoplasmatic reticulum is little developed (31). The slight enlargement of the vesicles of t endoplasmatic reticulum, occurring at the beginning of mustard oil action in pa ticular, should probably be regarded as a result of increased activity rather th as a pathological modification, since there is also a greater number of vesicles and their membranes appear covered with RNP granules more frequently. Since according to Miller (25) the free RNP granules are said to be responsible for the protein synthesis generally, while the ergastoplasm is linked with the production of certain proteins, e.g. secretions, these morphological findings may be regarded as reflecting a release of substances involved in the inflammatory process. As these substances can themselves exert a cell-damaging effect (24), besides other effects,they are probably of importance for the further modifications of the epidermis cell.

In the stage of massive vacuolization of the entire epidermis also a swelling of the vesicles of the endoplasmatic reticulum takes place, which must be regard as a passive osmotic process, as the production of energy is no longer sufficient to maintain the water balance in the cell.

After mustard oil as well as turpentine and cantharidin action, the nucleus shows a deposition of electron-impervious material on the inner nuclear membrane a phenomenon observed also by Mölbert (26) after hypoxydosis on liver cells and which he interpreted as an increase in activity. In preparations treated according to Feulgen, these depositions contain DNS.

While the often distinct formation of nucleoles suggest their active role in the cell happening, the degenerative modification of the nuclei after turpentine application, reflected also in the outcome of the Feulgen staining, indicates a severely damaged metabolism, as the nucleus is closely dependent on the cell in its energy requirement.

The separation of elements of the endoplasmatic reticulum is preceded by the increasing deposition of RNP granules on the outer nuclear membrane, as has been described also by Karasaka (20) for ectoderm cells of the triturus embryo, while generally the deposition of the RNP particles on the membranes of the endoplasmatic reticulum is said to take place only in the plasma (10).

The epidermis-cutis boundary shows in principle the same construction as all epithelial and endothelial basal membranes. But since the "basal membrane" of classic histology is composed of the submicroscopic double lamella and the contiguous collagen layersof the cutis, the term "adepidermal membrane" according to Salpeter and Singer (32) has been used.

The epidermis-cutis boundary proves to be a very sturdy strudure, in whose history disturbances are generally not ascertainable. In our studies we were able occasionally to observe the immigration of leukocytes; one can see that the leukocyte penetrates into the epidermis and that at this point the epidermis-cutis boundary is interrupted in its normal development. Connected therewith is the enlargement, observed on several occasions, of the adepidermal membrane, although this was n[illegible]r visible on cantharidin-treated skin, where at the same ti

the infiltration with leukocytes was missing. We would attribute this phenomen on the adepidermal membrane, not to general processes at the focus of inflammati but toa specific achievement of the leukocytes, since Kerby (21) found in leukocytes a lysozymoid enzyme which is capable of depolymerizing and dissolving mucopolysaccharides.

The keratinization increases as a result of the irritation according to the development of epidermis hyperplasia, while during the resting phase of the hair cycle the few-layered epidermis contains few tonofilaments and keratohyalin gran ules. The process of keratinization is a differentiation, not a degeneration pr cess. The energy required for it is to be supplied by glycolysis, since due to the increasing distance of the cells from the blood vessel-containing cutis the oxygen supply is hindered (4). The degeneration of the mitochondria in the stratum granulosum is seen by Fasske et al. (9) as the morphological expression of this. Glycogen is held by Bradfield (4) to be the energy reservoir. Actuall in pathological keratinization processes of the mucus membrane of the mouth ther is coincidence in glycogen occurrence and in the keratinization zone (9).

For the normal skin of the adult mouse, however, this coincidence does not exist, as the epidermis cells generally do not contain glycogen (28). Also unde our test conditions we were unable to show glycogen in the epidermis histochemic ly. Therefore, the energy supply must be sufficient also without additional sub strates. The occurrence of "lymph spaces" in the hyperplastic epidermis indicat better oxygen and nutrient supply, and the intact mitochondria in the stratum granulosum are possibly also of importance in answering this question.

The kerotohyalin granules are often in proximity of the nucleus, and usually mitochondria occur in their vicinity. Their high electron density is not caused by osmation, but occurs also after fixation in formol; their chemical nature and the mode of their formation are not yet clearly understood. An involvement of the mitochondria in the formation of the keratohyalin granules, assumed by Sheldon and Zetterquist (35) and Meneffee (23), was not observable in our prepar tions. There is often a very close spatial relationship with the tonofibrils, but the kerathyalin granules are formed at first as rounded structures independ-

ent of the tonofilaments, as was observed also by Brody (5) on guinea pig skin, in contrast to human skin (6).

The keratohyalin granules appear not very sharply delineated from the surroun ing, giving the impression as if RNP granules were deposited in the periphery (fig. 18a). This would be in line with the belief that the keratohyalin granule is surrounded by an RNP envelope (28). After hydrolysis with 1n HCl, both the RNP granules of the plasma and the granular depositions disappear, so that the grain appears very sharply delineated from the surrounding; besides, a loosening of the internal structure is seen (fig. 18b). But as RN-ase action had no effec this assumption is still not definitely proven.

In the upper stratum granulosum, as well as on the contact zones on the kerat hyalin granules, horn substances deposit, owing to which these lose their rounde shape and irregular forms result. During keratinization they become included in the keratin substance. This is particularly clear in transition cells, which co be observed occasionally after irritation - possibly due to a disturbed keratini tion (fig. 19). Brody (5, 6) describes "transition cells" also in normal skin.

The enlargement of the stratum corneum several days after the irritant applic tion must be regarded as a non-specific reaction, as it is caused also by cold (22) and various other influences. The increase of the horny strata is simply a concomitant of epidermis hyperplasia.

I am indebted to Miss Gisa Eichler for technical assistance, to the photo lab for photographic work.

Abbreviations used in the figures:

B = Stratum basale; C = Stratum corneum; G = Stratum granulosum; S = Stratum spinosum; aM = Adepidermal membrane; D = Contact zone; E = Epidermis-cutis bound ary; eR = Endoplasmatic reticulum; F = Tonofilament; I = Intercellulars; K = Keratin; Kg = Keratohyalin granule; M = Mitochondrium; N = Nucleus; P = RNP granule; V = Vacuole; Z = Cell membrane.

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auch durch Kälteeinwirkung [22] und verschiedene andere Einflüsse hervorgerufen. Die Zunahme der Hornschichten stellt lediglich eine Begleiterscheinung der Epidermishyperplasie dar.

Für technische Assistenz danke ich Fräulein Gisa Eichler, für fotografische Arbeiten dem Fotolabor.

In den Abbildungen verwendete Abkürzungen:
B = Stratum basale; C = Stratum corneum; G = Stratum granulosum; S = Stratum spinosum; aM = adepidermale Membran; D = Kontaktzone; E = Epidermis-Cutis-Grenze; eR = endoplasmatisches Retikulum; F = Tonofilament; I = Interzellulare; K = Keratin; Kg = Keratohyalingranulum; M = Mitochondrium; N = Zellkern; P = RNP-Granulum; V = Vakuole; Z = Zellmembran.

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Summary

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Резюме

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Summary

I. Quasdorf: Electron-microscopic examinations on the influene of irritants on the epidermis of mice

The changes in the epidermis of albino-mice as produced by the effect of mustard oil, turpentine oil an cantharidine are followed up by means of the electron-microscope in certain intervals. The irritants applied cause the depositing of DNA-substances on the inner nuclear membrane. In the course of time there occur differences.

Mustard oil: Within the first hour after application an increased number of pro-mitochondriae and vesicles of the endoplasmatic reticulum is to be oserved. Only after some hours a swelling of the mitochondriae becomes evident. This is accompanied by a considerable vacuolization of the cytoplasm. After a gradual retrograde change after some days a hyperplastic epidermis with the typical formation of the individual strata will develop.

Turpentine oil: The destruction of mitochondriae begins already after a very short period and usually lasts for several days. The vacuolization in the cytoplasm is only a moderate one, while the cellular nuclei appear to be rather clotted. The normalization is a very slow process; the marked epidermis hyperplasia is absent.

Cantharidine: The changes observed under our experimental conditions are minor, indeed. There occur disturbances on the mitochondriae after one to two hours, while the remaining cellular structures are next to normal. The retrogade change is effected very rapidly so that after a few days the epidermis will be hyperplastically changed.

The results are discussed with regard to the mechanism of action of the irritants applied. Besides, special attention is devoted to the question of the nature of kerathohyaline granulae.

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Tafel I

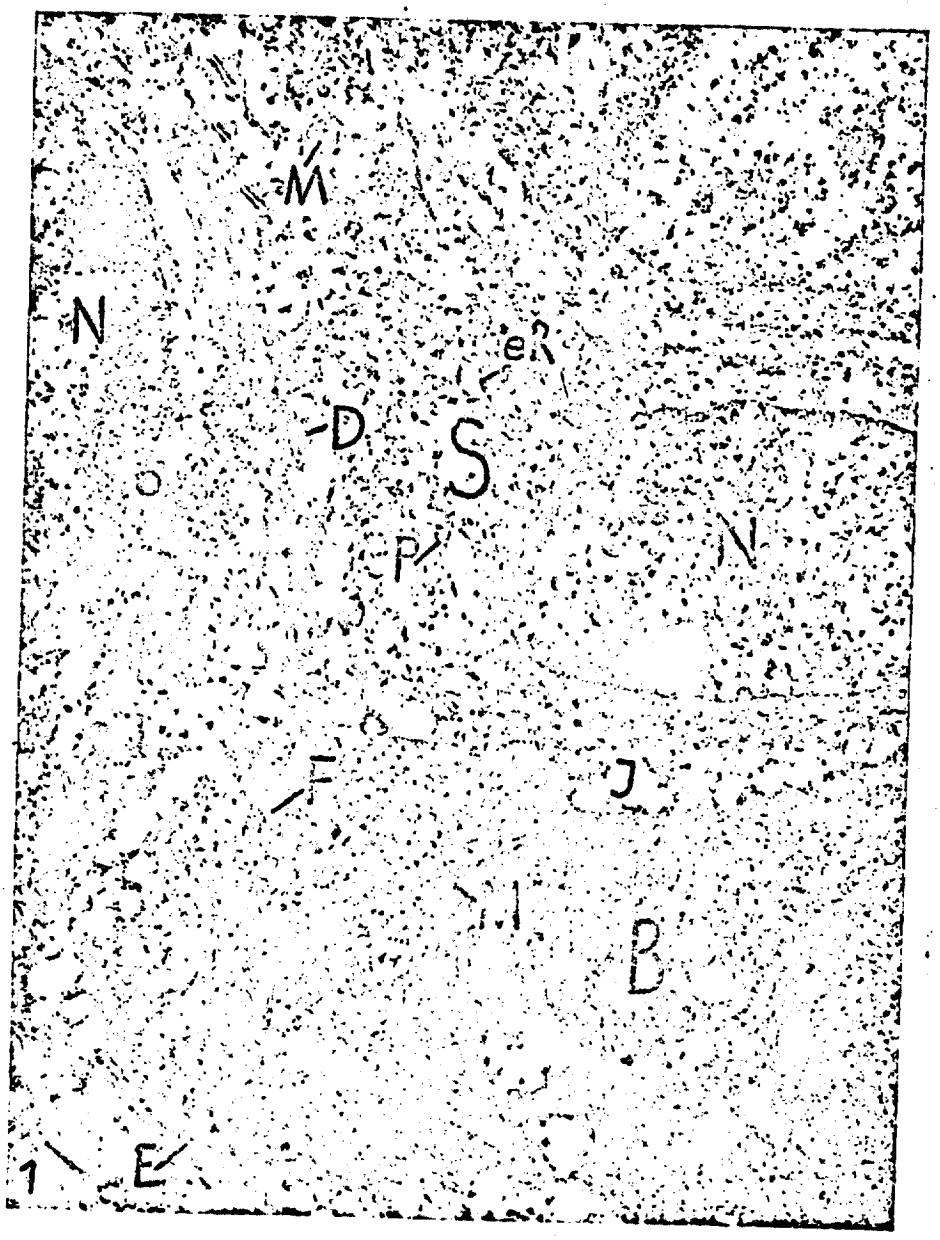

Abb. 1. Übersicht über die Strukturelemente der Epidermiszellen der Bauchhaut, 2 Std. nach Senföl-Applikation. 20000 : 1, Originalmaßstab 6000 : 1

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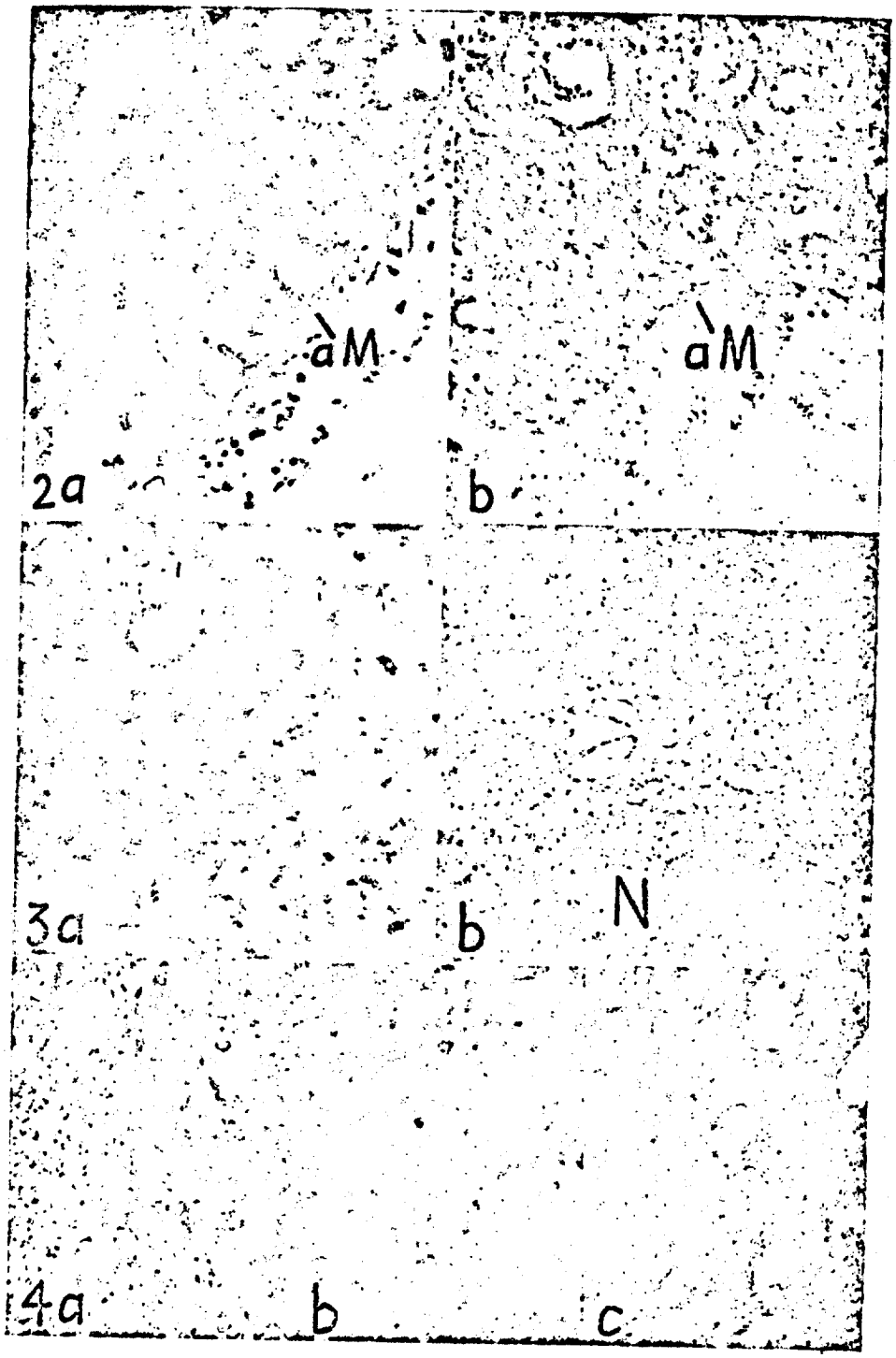

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Abb. 2. Epidermis-Cutis-Grenze, a) normal, 25000 : 1, Originalmaßstab 19500 : 1, b) einen Tag nach Senföl-Applikation, 25000 : 1, Originalmaßstab 6000 : 1

Abb. 3. Endoplasmatisches Retikulum, a) in der unbeeinflußten Epidermis, b) 2 Std. nach Senföl-Applikation, 30000 : 1, Originalmaßstab 6000 : 1

Abb. 4. Mitochondrien, a) normal, b) 2 Std. nach Senföl-Applikation, c) 6 Std. nach Senföl-Applikation, 30000 : 1, Originalmaßstab 6000 : 1

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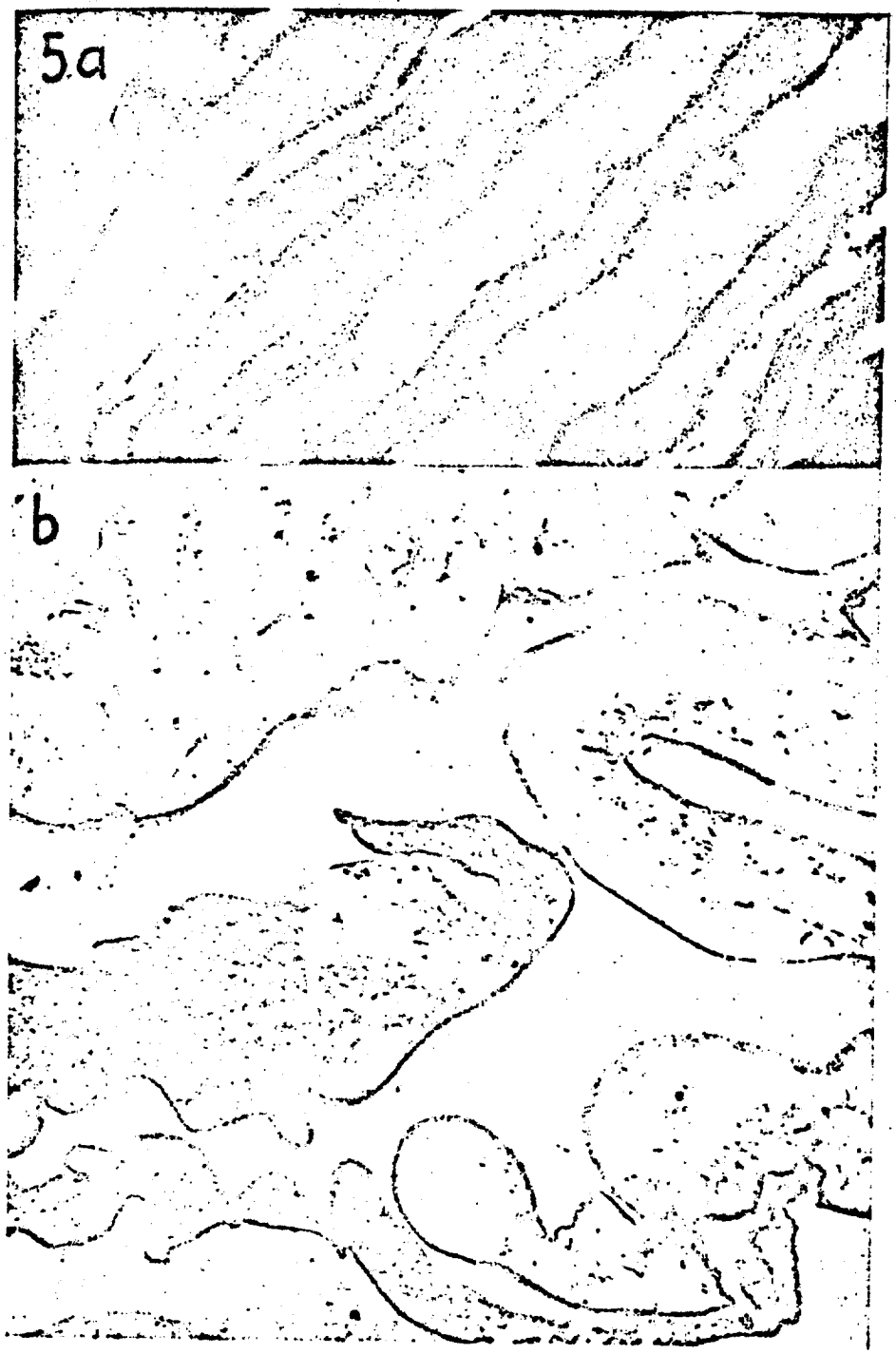

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Abb. 5. Stratum corneum, a) normale Hornlamellen, 40000 : 1, Originalmaßstab 19500 : 1, b) 2 Std. nach Senföl-Applikation, 20000 : 1, Originalmaßstab 6000 : 1

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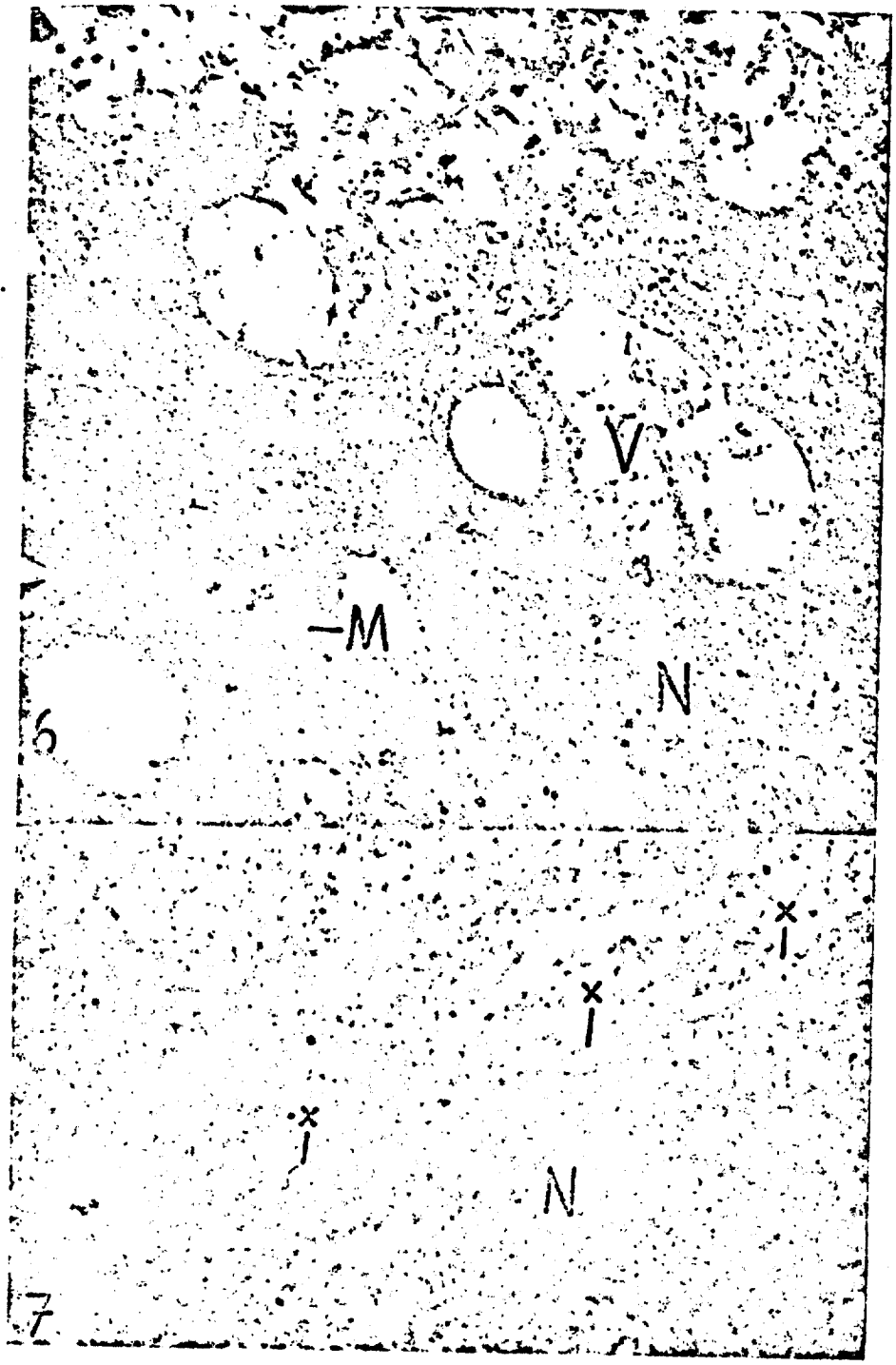

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Abb. 6. Bauchhaut, 2 Std. nach Senföl-Applikation. 20000 : 1, Originalmaßstab 6000 : 1

Abb. 7. Rückenhaut, 2 Std. nach Senföl-Applikation. 30000 : 1, Originalmaßstab 6000 : 1

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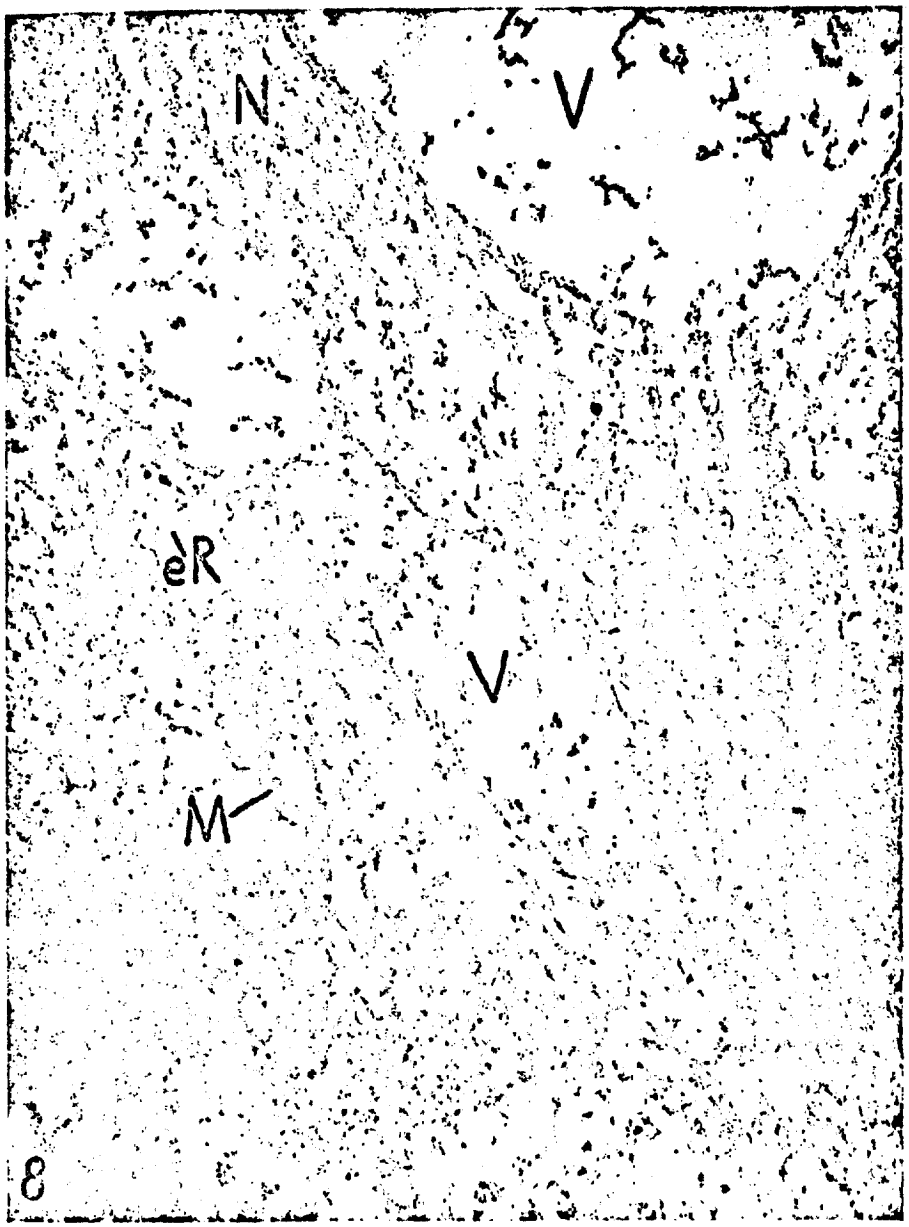

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Abb. 8. Bauchhaut, 5 Std. nach Senföl-Applikation. 20000 : 1, Originalmaßstab 6000 : 1

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Tafel VI

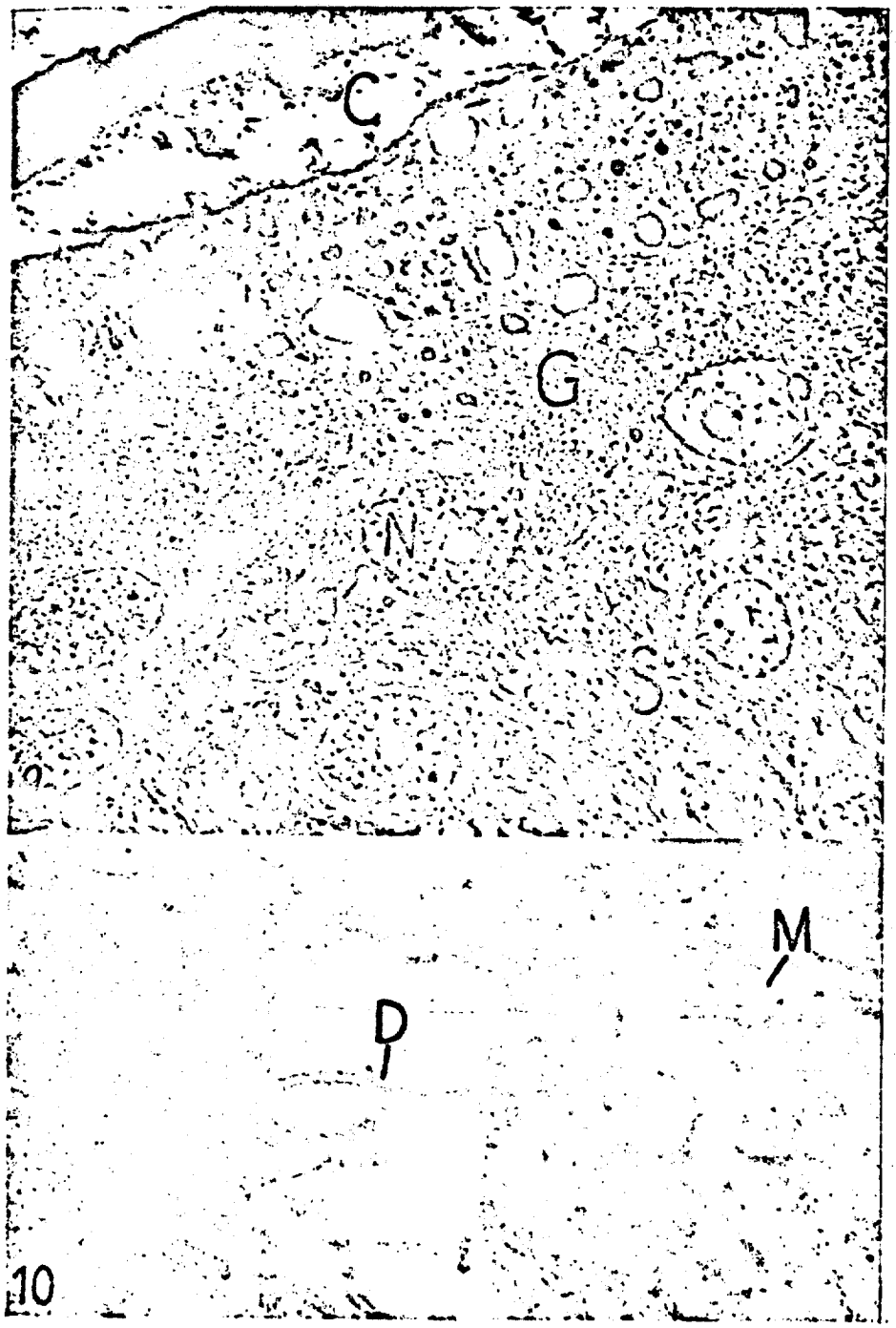

Abb. 9. Rückenhaut, 6 Tage nach Senföl-Applikation. 3000 : 1, Originalmaßstab 1340 : 1

Abb. 10: Rückenhaut, 6 Tage nach Senföl-Applikation. 30000 : 1, Originalmaßstab 6000 : 1

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Tafel VII

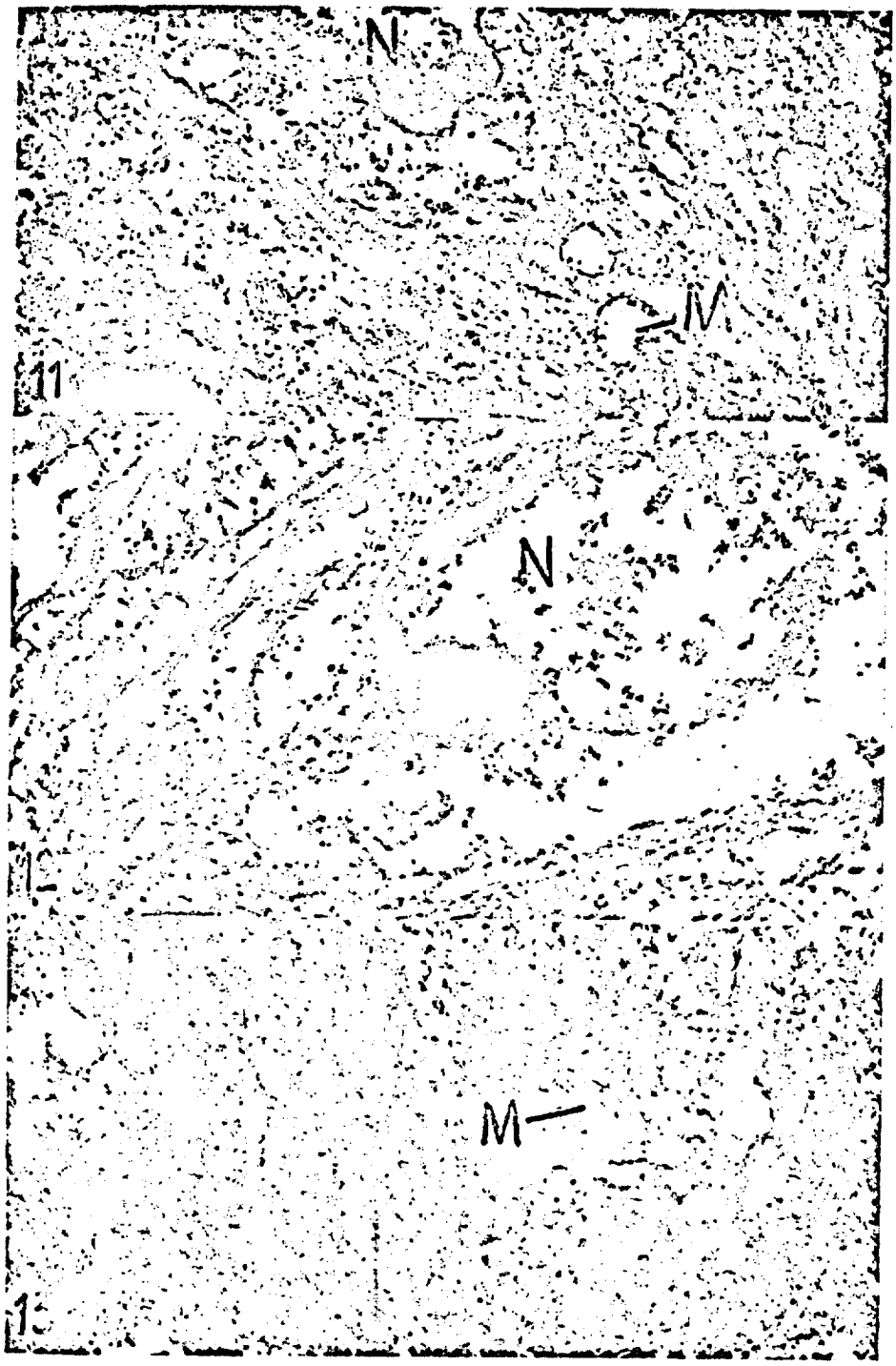

Abb. 11. Rückenhaut, 15 min nach Terpentinöl-Applikation. 30000 : 1, Originalmaßstab 6000 : 1

Abb. 12. Rückenhaut, 2 Std. nach Terpentinöl-Applikation. 20000 : 1, Originalmaßstab 6000 : 1

Abb. 13. Rückenhaut, 6 Tage nach Terpentinöl-Applikation. 20000 : 1, Originalmaßstab 6000 : 1

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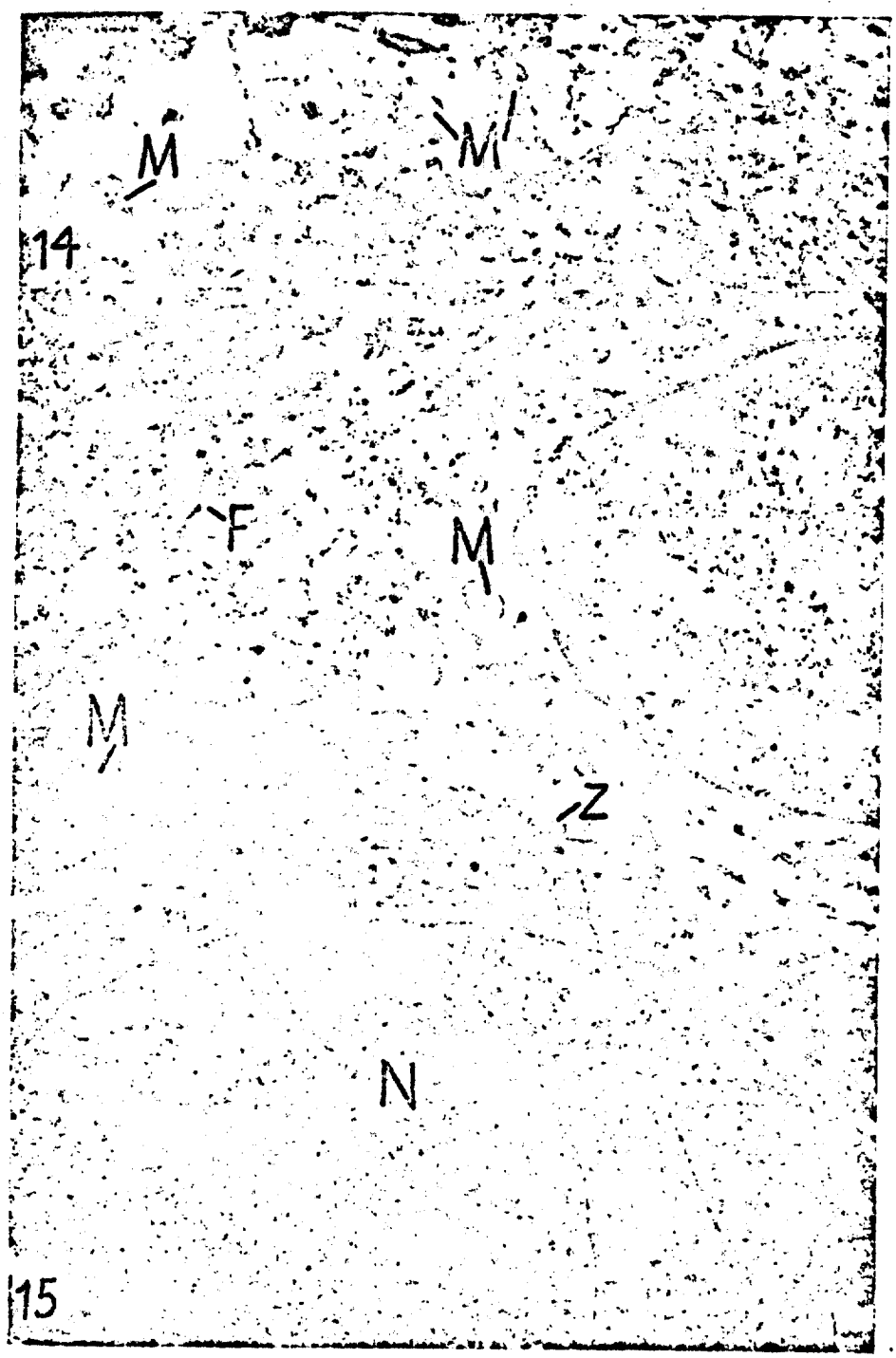

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Abb. 14. Rückenhaut, 1 Std. nach Cantharidin-Applikation. 30000 : 1, Originalmaßstab 9000 : 1

Abb. 15. Rückenhaut, einen Tag nach Cantharidin-Applikation. 20000 : 1, Originalmaßstab 6000 : 1

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Tafel IX

Abb. 16. Rückenhaut, einen Tag nach Cantharidin-Applikation. 8000 : 1, Originalmaßstab 2400 : 1

Abb. 17. Bauchhaut, 6 Tage nach Cantharidin-Applikation. 20000 : 1, Originalmaßstab 6000 : 1

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Tafel X

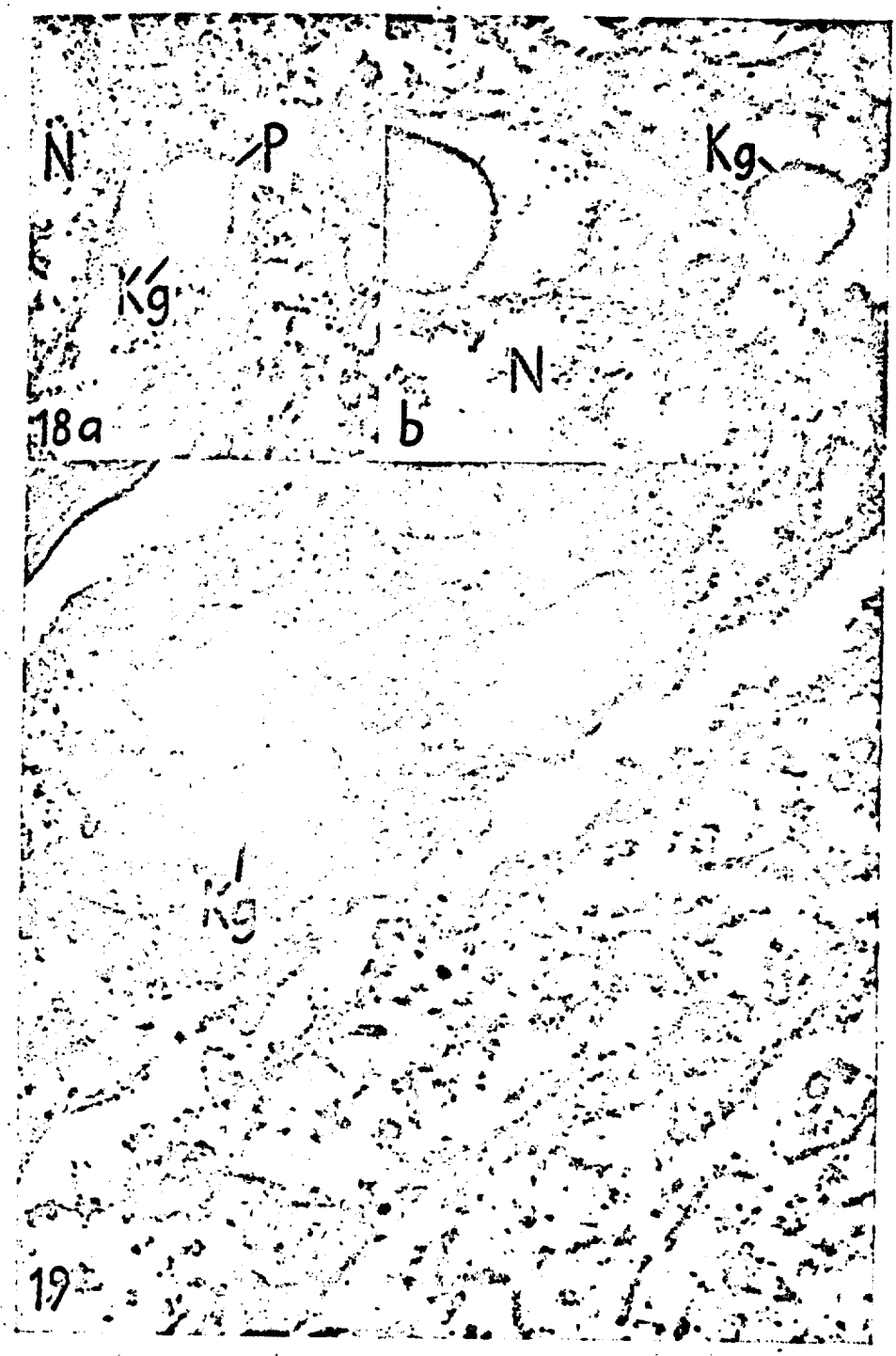

Abb. 18. Keratohyalingranula, a) ohne Vorbehandlung, b) nach Inkubation mit nHCl. 40000 : 1, Originalmaßstab 6000 : 1

Abb. 19. Übergangszelle. 25000 : 1, Originalmaßstab 6000 : 1

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(French publication, pp. 267-8)

EXPERIMENTS ON THE INCREASE OF CUTANEOUS RESISTANCE IN THE RABBIT

By J. Saudek

In the following experiments I applied the Ryback method; it consists, in principle, in the application on the shaved skin of the rabbit of small glass cylinders filled with revulsive substances (mustard oil, for example) at various concentrations. This application is done in parallel series, of three small tubes each, these tubes containing oil of mustard diluted to 50% with a different oil. The first series acts for 10 minutes, the second for 15 minutes and the third for 20 minutes. One hour after having removed the oil one observes on the skin a pronounced erythema accompanied with hemorrhages and edema. A deep necrosis appears the following day, in the form of a congested scab which forms a scar and heals only 15 days later. Every three days I repeated applications in series along the trunk, going from the front extremity to the rear extremity, spacing them approximately 3 cm apart. Starting with the third series I observed, on the part subjected to the experiments, an increasing resistance to the mustard oil; the intensity of the erythema, measured with a standard scale, decreased progressively and the necroses, deep at first, became more superficial, formed a scar more readily and healed more rapidly. Starting with the fifth or the sixth series there was no more necrosis. There remained but a slight dermatitis, accompanied by a desquamation which finally become imperceptible. In brief, after inflammation this region of the skin of the animal acquires a kind of immunity against irritations of chemical origin. It is curious to note that, in parallel with the reduction of the erythema, one observes an increase in the edema, which are more marked after the cylinders containing the oil are removed. I consider them as a protective reaction in opposition to the action in depth.

In order to more easily determine the importance of the reactions, the experiment was also performed on albino rabbits. The reaction was very pronounced and the ensuing necroses retained approximately the same depth until the fifth series of tubes. This indicates a much greater sensitivity of albino rabbits than pigmented rabbits. The dose of 50% mustard oil was too strong. After two month's

rest the necroses were completely healed; the skin, with the naked eye, appeare normal and the hair had grown back. I then repeated the same experiments on th same region of the skin of the animal; I observed a great resistance, as great as in pigmented rabbits after repeated application, or in rabbits subjected to ultra-violet rays for a fairly long time. Continuing the applications of musta oil an ever greater resistance is reached, going to almost complete immunity. (the skin of the opposite side, in the same animal, one observed only a decreased sensitivity, however not reaching complete immunity.

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ANAPHYLACTIC SHOCK FROM SKIN TESTING*

Two Cases—One Fatal

Oscar Swineford, Jr., M.D., University, Va.

ANAPHYLACTIC shock and death in human beings have been reviewed by Vaughan.[1] Thomas and Fenton[2] have reported three deaths and four systemic reactions from pontocaine used in endoscopy. Sinapoidin caused shock in Phillips' case.[3] There have been two other reports,[4, 5] since 1938, of shock after intracutaneous injections. One recent death from 10 grains of aspirin will be reported separately. In 1941,[6] evidence was offered to support the belief that anaphylactic shock from skin testing was the result of exquisite sensitization to one or a few allergens rather than to the accumulated effect of numerous strongly positive reactions. A rapidly developing huge local reaction had, until then and later, given warning of each impending troublesome systemic reaction to skin tests or to injections of allergens.

Ratner[7] and Rowe[8] described anaphylactic shock from intracutaneous test injections from which there were negative local skin reactions.

The present report is of two cases, one fatal, which occurred before skin tests had become strongly positive.

CASE REPORTS

Case 1.—A white, married man, aged 49 years, had had asthma for four years. He also had had urticaria, poison ivy, and hay fever. Four severe asthmatic attacks occurred two weeks before admission after several months of comparative freedom from symptoms. The asthma was perennial with exacerbations in early spring and in October or November and was accompanied by purulent sputum and postnasal discharge. Adrenalin always had relieved previous attacks.

Mustard, catsup, mayonnaise, and cabbage caused distention and burning of the throat; dust from hay, feeds, grains, and house caused asthma and hay fever; temperature changes, dampness, exercise, and excitement caused wheezing.

The remainder of the history was irrelevant.

On physical examination the essential findings were a typically pale, boggy, and swollen nasal mucosa. Numerous asthmatic râles and ronchi were heard in the lungs.

Routine examinations of urine and Wassermann and Kahn reactions were negative. Blood examinations were not reported.

The day after admission, after a comfortable night, the patient was sent to the allergy laboratory for routine skin testing. He was wheezing moderately and was slightly cyanotic but was not acutely uncomfortable. Symptomatic treatment had been withheld that morning. Preliminary cutaneous tests with 72 commonly reacting allergens were negative. After 56 routine intracutaneous food extracts and eight or nine intracutaneous inhalant extracts had been injected he complained of not feeling well. There was no cough or dyspnea immediately. He was given two doses of adrenalin of 3 minims each. A tourniquet was applied

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above the test sites. Rather suddenly, in a minute or two, perhaps ten minutes after the tests were started, he started gasping for breath and became extremely apprehensive and cyanotic. Within another minute or two he was comatose. The time between his statement that he did not feel well and the advent of coma and extensive acute emphysema was not more than five minutes. He was given 0.5 Gm. of aminophylline intravenously, oxygen by nasal catheter, artificial respiration, and additional adrenalin. He was placed in the mechanical respirator. However, before aminophylline administration was completed, he seemed to be dead. His heartbeat could not be heard nor could his pulse be felt within fifteen minutes after he had entered the laboratory. He did not respond to any of the efforts to resuscitate him.

The catastrophe occurred before any of the five or six positive food reactions had become stronger than 1 plus or 2 plus. They did not get stronger, and there was no time to identify them. The few inhalants did not react. By the time the emergency measures had been completed the reactions had subsided. A post-mortem examination was not permitted.

Case 2.—A white, married woman, aged 49 years, had had asthma, intermittently, for fifteen years. Attacks occurred about twice a month with no seasonal variation and were frequently associated with hay fever and urticaria. There was no sinus infection but there was some eczema of the face.

Asthmatic attacks followed ingestion of onions, tomatoes, and mustard; asthma, cough, or vasomotor rhinitis was induced by dust, chicken feed, horses, tobacco smoke, face powder, perfume, and roses; and asthma followed exposure to cold, exercise, fatigue, or excitement.

On physical examination, moderate wheezing obscured the heart sounds, the blood pressure was 125/80. Numerous other symptoms and physical signs were irrelevant. There were no foci of infection in ears, nose, or throat.

X-ray films of the chest were reported as normal. Routine studies of urine, stool, blood count, and Wassermann and Kahn reactions were normal.

Preliminary cutaneous tests with 72 commonly reacting allergens were negative. Fifty-six routine intracutaneous food extracts and 8 or 9 intracutaneous inhalant extracts had been injected when she complained of thickness of the tongue. Moderate wheezing began in one or two minutes. In another minute or two she became extraordinarily apprehensive and was thrashing about wildly. Dyspnea increased alarmingly. During the next two or three minutes, five 3-minim doses of adrenalin were given, one of them intravenously. She was given 0.5 Gm. of aminophylline intravenously. Within two or three minutes the respiratory difficulty was no longer alarming, but she continued to be apprehensive. She vomited several times and spat up large quantities of mucus. Immediately after administration of adrenalin and aminophylline, the blood pressure was 65/55 in the right arm. It remained between 65/55 and 80/65 until an intravenous saline infusion was started ten or fifteen minutes later. The blood pressure rose to 118/80 after 150 c.c. of saline had run in. The pulse, which had been about 130 beats per minute, slowed to about 100, and her chest became almost free from ronchi. She was returned to the ward where she relapsed into shock, with a blood pressure of 65/50, without appreciable asthma. Adrenalin in oil, ephedrine, and another infusion brought her out of shock promptly. Her recovery was uneventful.

Five or six intracutaneous food reactions could have been graded 1 plus or 2 plus when she became so ill that there was no time to identify them. By the time she seemed out of danger the local reactions had subsided. None of them became strongly positive. The few inhalants did not react.

Four days later scratch tests were done with all of the foods which had been injected intracutaneously originally. These gave weakly positive cutaneous reactions to American cheese, egg, vanilla, citrus fruits, rye, rice, beef, chicken, milk, pecan, English walnut, sweet potato, cucumber-squash-melon, cow hair, and feathers. There were strong cutaneous reactions to wheat, barley, lamb, lettuce-salsify, cabbage-cauliflower-kale-broccoli-brussel sprouts, beans, asparagus-onion, white potato-tomato, carrots-celery-parsnip, and olive. There was a huge cutaneous reaction to the group containing mustard-turnip-watercress.

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There were strong intracutaneous reactions to mattress and rug dusts, asthma powders, cotton linters, *Pyrethrum*, *Fusarium*, and karaya gum. There were numerous negative, questionable, and weak cutaneous and intracutaneous reactions which need not be detailed.

After the huge cutaneous reaction to mustard, etc., she remembered that, when she worked in a restaurant, the other waitresses had had to handle dishes with mustard remnants on them because the taste, touch, or smell of mustard would cause asthma or a rash promptly. Because of this the shock was attributed to the intracutaneous test with mustard, although no attempt was made to prove it. The mustard-turnip-watercress group was not in the preliminary cutaneous test substances.

She was discharged from the hospital, symptom-free, on the usual initial program of avoidance of positively reacting foods and inhalants plus the use of dustproof mattress and pillow cases and symptomatic remedies.

DISCUSSION

The technique for testing these patients was the same as that used on about 7,000 patients in the past ten years—a total of nearly one million individual tests. Several other patients, after a huge prodromal local reaction, had had some urticaria or wheezing and one had had severe asthma after intracutaneous tests. Each, however, had been relieved promptly by adrenalin and a tourniquet above the local reaction.

These two experiences, plus those of Ratner[7] and Rowe,[8] have not altered our belief that constitutional reactions are the result of exquisite sensitization to one or a few allergens.

These severe reactions prove conclusively that a routine of scratch tests with 60 or 70 common reactions, as was then in use in this and other clinics, is an inadequate precaution against disaster. Every intradermal test should be preceded by the less sensitive scratch test.

SUMMARY

1. Two cases of anaphylactic shock from intracutaneous skin tests with food extracts are described. The food which caused the fatal reaction was not determined. The nonfatal reaction was thought to be due to mustard.

2. In both, anaphylactic shock occurred before there was time for the skin reactions to become strongly positive.

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(pp. 69-74, German)

Experimental Studies on Alterations of the Mucus of the Upper Respiratory Tracts on Inhalation of Toxic Gases (*)

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(Received November 37, 1933)

The matter of toxic gases is a problem which requires study not only in times of war, but now also in peacetime. There are, therefore, many pathological and anatomical papers on it. During the World War and after, many such reports were published, but although some of them are based on experiments with various gases they deal mostly with alterations in the trachea and lung. Papers reporting gen erally on pathological changes of the nasal cavity as the entrance-way to the respiratory tracts and of the upper respiratory tracts are extremely rare. Ther are only a few, with respect to gases, that discuss changes of the nasal mucus b dichlorodiethyl sulfite, and, with respect to non-gaseous substances, the same changes due to chromic acid particles. Besides, only occasional, short remarks about our problem are found in the textbooks of Denker & Kahler, Fluyr and other

This has prompted the author to make experiments with 9 types of toxic gases which are of importance not only in war but also in peace-time in the chemical industry and about which no detailed reports, or none at all, were available, i. with chlorpicrin, cyanogen bromide, allyl mustard oil, paradichlorobenzene, carbon disulfide, amidobenzene, iodine, bromine, sulfur dioxide, and to make system atic studies on their effects on the upper respiratory tracts.

As test animals healthy guinea pigs of a body weight of about 400 g were used

Test arrangement

The test animals were placed in large glass vessels holding 2500 cc. Then rapidly evaporating chloropicrin, carbon disulfide and bromine were dropped on f ter paper freely suspended in the glass vessel and allowed to vaporize. For othe difficultly evaporating chemicals, there was placed in the bottom of the large glass vessel in which the animals were contained, a metal cylinder in which a

small glass dish was placed. In this dish the difficulty evaporating chemicals were heated by an alcohol lamp placed under it and thus caused to evaporate.

The concentration of the gases was different depending on their type, but different mixture ratios with air from 1:5000 to 1:2,500,000 were prepared. The animals were let breathe these mixtures for 5, 10, 15 or 20 minutes. The concentration of the gases and the time during which the animals were allowed to breath them were varied according to the degree of toxic effect of the gases.

The following table clearly shows these relationships, stating the concentrati of the gas used and the shortest and longest duration of use of each gas.

Chloropicrin	1/50,000 - 1/2,500,000	5-10 min
Cyanogen bromide	1/125,000 - 1/2,500,000	5 min.
Allyl mustard oil	1/25,000 - 1/500,000	5 min.
p-Dichlorobenzene	1/5,000 - 1/2,500,000	20 min.
Carbon disulfide	1/10,000 - 1/250,000	5 - 10 min.
Amidobenzene	1/8,333 - 1/250,000	10-20 min.
Iodine	1/50,000 - 1/2,500,000	10 min.
Bromine	1/25,000 - 1/500,000	5 - 10 min.
Sulfur dioxide	1/25,000 - 1/2,500,000	3 - 5 min.

The test animals were exposed to the toxic gases in the concentrations stated in the table and for the times mentioned therein, either once or repeated 2 to 20 times. Then the changes in the mucus of the upper respiratory tracts wer investigated for 2 days to 6 months.

The total number of guinea pigs used for these tests was 250. Among them 34 died in their cages after having been used once or several times for tests. As they usually died during the night, so that the exact time of death was not recorded, they were not taken into account. The number of microscopically examined animals, therefore, is 216.

Clinical findings

The test animals closed their eyes 5-15 seconds after the chemicals used for the test had begun to evaporate, and tears began to run from their eyes.

With the paralyzing gases, such as carbon disulfide and amidobenzene, this

sympton appeared much later, 2-3 minutes after start of evaporation. Also, copious nasal secretion and reddening of the vestibulum nasi and of the nasal mucus were symptoms appearing with all gases. There were animals whose nasal secretion was so abundant that it dripped from the nose almost continuously. Some animals also had saliva running from the mouth. They showed unrest, anxiety, oppression, and in particularly severe cases they became frantic. Quickened respiration occurred in early cases 1-3 minutes after start of evaporation, follo ing this also dyspnea. The time of occurrence of these phenomena and their degre were generally in direct proportion to the concentration of the gas and the lengt of exposure. Also differences depending on the type of the chemicals were observable. Chloropicrin and cyanogen bromide caused the earliest and strongest symptoms, followed, in degree of action, by iodine, bromine, sulfur dioxide gas, while carbon disulfide and amidobenzene brought about the most delayed and weakest effects. Nausea and vomiting could not be said to be general symptoms. Nor was the vomited matter always the same.

Among the animals which had shown at first unrest, excitation, even frenzy, many gradually calmed down and fell into a stupor. Finally the animals rolled back and forth, lay on their side and were unable to stand up again. With all chemicals, cases of asphyxiation occurred at high concentration and long exposur For mustard oil and sulfur dioxide we should mention in particular their propert to cause sneezing.

Also in the state of the animals after the tests differences were caused by the above-mentioned differences in the type of the chemicals, their concentration and time of application. Some animals became limp in the end, lay on their side stretched out, had convulsions, and finally died; others showed improvement of all symptoms by and by. Among those recovering there were differences in degree While it was possible to observe different states in the sneezing spells, the nausea, vomiting, decrease of the respiration frequency, abating of the dyspnea, it seemed that in general all symptoms improved in the animals on which tests

with gases of low concentration were made several times in succession - although it must be admitted that the movements of the animals in the 24 hours between tests were somewhat limp as compared with the control animals. In general the appetite of the animals and the relative increase of body weight were slight during the tests or thereafter.

Histological findings

There were great differences in the alterations depending on location. The alterations in the mucus of the septum nasi, the regio respiratoria and olfactor the canalis nasopalatinus and the meatus nasopharyngeus were relatively great; especially at the septum nasi and the regio respiratoria and olfactoria extremel great changes were observable. At the septum nasi the changes were severe in th upper anterior and became gradually less toward the lower posterior. Also in th regio respiratoria and olfactoria, especially in the front portion, i.e. in the space between regio olfactoria and septum nasi, and in the transitional portion from the regio respiratoria to the regio olfactoria, at the forward edge of the turbinated bone, considerable alterations appeared. In the parts of the side walls of the nasal cavity covered by squamous epithelium, the changes were relative slight. In the canalis nasopalatinus and meatus nasopharyngeus the changes increased gradually toward the back. In the vestibule of the nose, Jacobson's organ, canalis nasolacrimalis, sinus paranasales, pharynx and larynx, the change were relatively slight in most animals. In the canalis nasolacrimalis, especial upper posterior, great changes were seen. In Jacobson's organ especially the epithelium of the outer wall was affected. The concha nasalis inferior along with the regio respiratoria and olfactoria had undergone the greatest changes. These were particularly severe in the lower half of the outer side, while in the upper half of the inside and outside they were relatively slight. Naturally the changes were great at all parts toward the path of inhalation, directly exposed the inhaled air, but when large quantities of chemicals were used and when the exposure times were long, the above-mentioned local differences in degree of al-

teration were hardly perceptible. Inside the nasal cavity, of course, and into the deeper parts of the throat and windpipe, all parts had suffered great altera tions. Also depending on the type of gas, diverse differences in the degree of alteration were produced: Chloropicrin, cyanogen bromide, bromine, iodine cause the greatest changes; mustard oil the next greatest; amidobenzene, sulfur dioxid carbon disulfide, paradichlorobenzene relatively slight ones. Also among gases the same kind, the irritant gases iodine and bromine seemed to damage the tissue exceedingly severely as compared with sulfur dioxide, and differences were observable also in the effects which each of these gases caused.

The main alterations were: Ulceration, necrosis, and decay of the epithelial cells, all kinds of degenerative alterations, formation of defects, vacuoles in the epithelium and cysts; dilatation of the blood vessels and hyperemia were found in all animals; also cases of hemorrhage were not rare. In the submucous tissue one could frequently see infiltration of round cells, neutrophilic polynuclear leukocytes, cosinophilic cells, etc., rarely also large mono-nuclear round cells were observed. But depending on the chemicals, there were cases in which cell infiltration was relatively strong and the dilatation of the blood vessels and hyperemia relatively mild. In other cases, on the contrary, there was quite definitely great dilatation of the blood vessels and hyperemia. In many cases, after repeated tests, infiltration was observable and occasionally also plasma cells occurred. The submucous connective tissue became loose in light cases, while it showed an edematous state in severe cases. All these symp oms appeared mainly when large quantities of chemicals had been applied over a long time. With respect to the chemicals, these alterations were most obvious with bromine, and with respect to location, they were particularly marked in the regio respiratoria and olfactoria, in the canalis nasolacrimalis and meatus naso pharyngeus. Also the gland tissue was often altered, showing enlargement and swelling of the gland cells, tendency to intense stainability, and in severe cas decay and necrosis of the cells. There were also severe cases of dilatation of the gland ducts. Such symptoms were observed often when using large quantities

of chemicals over a long time or when the tests were repeated several times. Some time after the test, various pictures of regeneration and multiplication of the epithelial cells could be observed, but these pictures were different depending on the length of time passed and the magnitude of the alteration suffered. No changes were observed on bones, cartilage, periosteum and perichondrium.

Vacuolization in the epithelium and cyst formation occurred most frequently 3-4 days to 1 week after the tests. Also infiltration of submucous cells was found in some cases where more than 1 month had gone by since the test.

(*) The original was published in Aichi Igakkai Zasshi 40 (1933), 472-556 (in Japanese).

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Ind. Jour. Med. Res., 53, 10, October, 1965.

TESTING CARCINOGENICITY OF EDIBLE OILS.

Part II.

MUSTARD OIL

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(Received for publication, May 25, 1965.)

THE mutagenic property of the mustard oil (Allyl isothiocyanate) was reported long back by Auerbach and Robson, 1944 ; Sikka and Swaminathan, 1956 ; Swaminathan and Natrajan, 1956, 1959. These workers have also described tumour production by mustard oil in allium root tips. This oil is widely used in Bengal and U.P. in the diet and is also commonly used for massage. To investigate the possible carcinogenic effect of mustard oil on animal tissues, a series of experiments were undertaken on laboratory mice.

MATERIAL AND METHOD.

Two samples of mustard oil were used for this study. One was obtained from the Indian Agricultural Research Institute, New Delhi, and the other was a locally available preparation—tinned mustard oil (Raj brand) from Raj Oil Mills, Bombay.

A total number of 86 hybrid mice (XVII × C57 black) was used as test animals. The treatment was started between the age of 10 and 12 weeks and the animals were observed till death or were sacrificed when they looked weak and emaciated.

Routes of administration.—The oil was administered by four different routes :

(*i*) Cutaneous application (*ii*) Subcutaneous injection
(*iii*) Intraperitoneal injection, (*iv*) Feeding by stomach tubing.

Experimental groups were arranged as follows :—

To test mustard oil sample received from I.A.R.I., New Delhi :

Group I.– Fourteen mice were fed 0·05 ml. of oil daily (except Sundays) by stomach tubing for 3 months, and observed till death.

To test Raj brand mustard oil :

Group II. – Daily cutaneous applications of oil were given by No. 5 camel hair brush on the interscapular region of 12 mice.

Group III.—Croton oil was used as a co-carcinogen along with mustard oil in a group of 12 mice. Cutaneous application of mustard oil was given four days per week and 3 per cent croton oil in liquid paraffin was given once a week. Both the groups II and III received the treatment till death.

Group IV.—Four subcutaneous injections of 0·5 ml. of mustard oil were given to 12 mice at monthly intervals. Total dose was 2 ml. per animal.

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Group V.—Five intraperitoneal injections of 0·2 ml. of oil were given to 13 mice at monthly intervals. Total dose was 1 ml. per animal. The animals from groups IV and V were kept under observation till death.

Group VI.—Ten mice were fed daily 0·05 ml. oil throughout their life except on Sundays.

Group VII.—An untreated control group of 13 mice was observed till death.

These animals were sacrificed when they appeared weak and emaciated. The site of treatment and all visceral organs including gonads were observed for gross abnormalities. Suspected tissues and the treated site were fixed for histo-pathological studies.

Experimental findings.

In the cutaneous application groups (groups II and III) no epidermoid tumour was developed. Skin was normal. Slight to moderate epidermal hyperplasia was seen in group III, where co-carcinogen was used.

In the subcutaneously injected group (group IV), deposition of oil or its metabolites was seen as observed before in the animals injected with pea-nut oil (Gothoskar and Ranadive, 1965). No tumour of subcutis was developed during 100 weeks of observation period.

In the intraperitoneally injected group (group V), no lesions were seen in the visceral organs.

In the mice with continuous feeding of oil (group VI) one of the ten mice developed papilloma of the stomach wall at the cardiac end. In the mice fed with oil, supplied by Indian Agricultural Research Institute, New Delhi (group I) for three months, three of the fourteen mice developed stomach papillomas (Plate LX). In untreated control mice, also, one of the thirteen had a papilloma of the stomach wall. Microscopic study of the liver lesions found in this series showed fatty degeneration, necrosis and patches of inflammatory cells. Such liver lesions were also present in the untreated control mice. Other abnormalities found in this series were atrophic spleen and enlarged lymph nodes (*vide* Table).

Discussion.

Experimental testing of mustard oil administered by four different routes, namely cutaneous application, subcutaneous injection, intraperitoneal injection and oral feeding, was undertaken and the animals were observed for sufficiently long period of over 2 years. Few papillomas of the stomach wall, inflammatory lesions of the liver, atrophy of spleen and enlargement of lymph nodes observed in some experimental groups of mice were also found in the untreated mice. There was thus no evidence of any malignancy at the site of treatment or in the remote organs, that can be attributed to the administration of oil. These observations confirm the findings of Visser and Ten Seldam (1938) and of Larionow and Soboleva (1938) on albino mice (quoted by Hartwell, 1951).

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TABLE.

Summary data of mustard oil testing for carcinogenicity

Sample of oil.	Route of administration.	Number of animals.	Abnormalities: Skin.	Liver (necrosis and fatty changes).	Spleen (atrophy).	Stomach (papilloma at the cardiac end).	Lymph node (enlarged).	Malignancy.
I.A.R.I. New Delhi sample.	Control ...	13	..	1	6	1	6	..
	Oral administration for 3 months	14	1 Papillomatous growth on eye lid	2	7	3	6	..
Raj Brand, Bombay.	Cutaneous application	12	..	1	..	..*	1	..
	Cutaneous application with co-carcinogen	12	6 Hyperplastic oil deposition	..	1	..*	..	..
	Multiple subcutaneous injection	12	12	..	4	..*	..	..
	Multiple intraperitoneal injection	13	..	..	3	..*	..	..
	Continuous feeding ...	10	..	..	2	1	2	..

* Stomach wall not examined.

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The mutagenic and tumorogenic activity of the mustard oil as reported by Swaminathan and Natrajan (*loc. cit.*) appears to be specific to plant tissues only. Testing oil for the mutagenic property direct on animal cells would perhaps throw more light on the subject.

SUMMARY.

Mustard oil has been tested for its carcinogenic activity on XVII × C57 (black) hybrid mice by four different routes : (*i*) Cutaneous application, (*ii*) subcutaneous injection, (*iii*) intraperitoneal injection and (*iv*) oral administration. There was no evidence of malignancy due to oil at the site of treatment or in the remote organs even though the animals were observed for their complete life span of over two years.

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*(350) Perfume and Flavor Chemicals. 1965. Item 98. S. Arctander.

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98: ALLYL-iso-THIOCYANATE

Allyl-*iso*-rhodanide.
Allyl Mustard oil.
Mustard oil, artificial.
Allyl-*iso*-sulfocyanate.
Allyl senevol.
Allyl thiocarbonimide.

$CH_2{=}CH{-}CH_2{-}N{=}C{=}S$

$C_4H_5NS = 99.15$

Colorless mobile liquid. B.P. 151-153° C. Sp.Gr. 1.025.

Slightly soluble in water, miscible with alcohol and flavor materials. The oil turns darker yellow upon exposure to air and daylight.

Extremely penetrating, pungent and stinging odor. This chemical is a powerful lachrymator, and inhalation of its vapors may cause serious damage to human lungs. The liquid and its vapors produce blisters on the human skin. A few grams may kill a man, if the oil is taken internally.

In spite of its apparent power and pungency, this chemical can be tolerated in surprisingly high concentration as a flavor chemical. However, the pungency is perceptible down to a level of 1-3 ppm in aqueous solution.

Used quite extensively in flavor compositions, particularly along with vinegar (pickled products). Also in meat and spice flavors. Concentration in pickled products may be as high as 80 ppm.

This chemical is very unstable. It is decomposed under influence of air, light, water, metals, etc. and even alcoholic solutions are unstable.

Prod. from Allyl iodide and Potassium sulfocyanide. This chemical is the chief component of the volatile oil of black Mustard seed, and can be isolated by enzymatic breakdown of the glycoside in the seed.

G.R.A.S. F.E.M.A. No.2034.

3-115; 7-21; 65-734; 66-946; 72-27; 77-213; 85-106; 86-123; 90-841; 100-37; 140-177; 160-1066;

Guenther, E. 1952. Oil of black mustard seed. The Essential Oils. VI. 55-56.

OIL OF BLACK MUSTARD SEED

(Oil of Mustard)

Essence de Moutarde *Aceite Esencial Mostaza* *Senföl*
Oleum Sinapis

The seeds of *Brassica nigra* (L.) Koch and of *Brassica juncea* (L.) Czerniacw (fam. *Cruciferae*) contain fixed oils of mild taste, which can be isolated by expression of the seeds in hydraulic presses. The chemical composition of these fatty oils resembles that of oil of rapeseed (*Brassica napus* L.).

Much more important than the fixed oils from the point of view of flavor are the volatile oils derived from the seeds of *Brassica nigra* and *Brassica juncea*. Both of these varieties are classified as "Black Mustard" in the United States Pharmacopoeia and the United States Dispensatory. The volatile oils are not contained in the seeds, in the free state, but in the form of glucosides. To isolate them from the seed, it is necessary to remove the fixed oil by expression, to submit the pressed cake to fermentation, and to distill the fermented mass (see below).

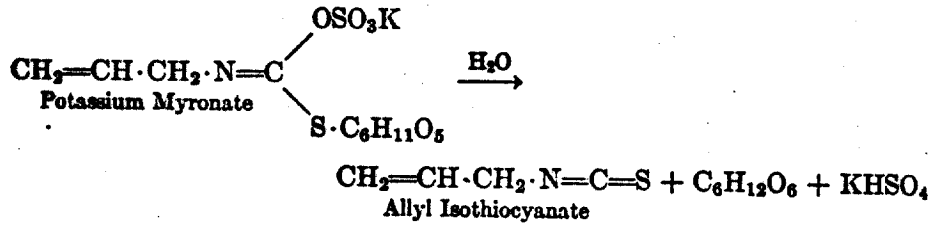
The volatile oil derived from black mustard seed (*Brassica nigra* or *B. juncea*) consists almost entirely of allyl isothiocyanate. This compound can also be prepared synthetically. In fact, most of the mustard oil or "Allyl Mustard Oil" offered on the market today consists of the synthetic product. The natural oil has lost much of its former importance. Official standard works in the United States recognize both the natural and the synthetic oils as "Volatile Oil of Mustard" or "Allyl Isothiocyanate." It is required, however, to specify on the label whether the product is natural or synthetic. In both cases the actual content of C_3H_5NCS in the oil should not be below 93 per cent.

For full information regarding natural and synthetic allyl isothiocyanate the reader is referred to Vol. II of the present work, p. 734. Suffice it here to discuss the production of the natural oil.

Brassica nigra (L.) Koch, the "Black Mustard," is a native of central and southern Europe and western temperate Asia. The plant, an annual, attains a height of 2 ft. or more. It is cultivated extensively in countries of temperate climate, particularly Holland and Italy, the seed from Holland being considered of best quality.

Brassica juncea (L.) Czerniaew, also called "Brown Mustard," "Indian Mustard" or "Rai," is cultivated in northern India, in Astrakhan, Sarepta, and other parts of southern Russia. The seed of this species closely resembles that of *Brassica nigra*, hence both are accepted as "Black Mustard" in the United States. The volatile oils derived from the seeds of the two species are practically identical.

Production of the Volatile Oil.—As was pointed out above, allyl isothiocyanate does not occur in the seed as such, but in the form of a glucoside, viz., sinigrin (potassium myronate), which, in the presence of water and a ferment (viz., myrosinase), is hydrolyzed to allyl isothiocyanate, *d*-glucose, and potassium bisulfate:



According to Wrede, Banik and Brauss,[1] the empirical molecular formula $C_{10}H_{18}NS_2KO_{10}$ of the glucoside sinigrin (m. 128°) applies to the crystalline compound, which contains water. The formula should be resolved into $C_{10}H_{16}NS_2KO_9 + H_2O$. On boiling of the methyl alcoholic solution, after addition of absolute alcohol, sinigrin (m. 179°), free of water, will be obtained. This latter form of the glucoside is readily soluble in water. Hérissey and Boivin[2] succeeded in preparing pure sinigrin $[\alpha]_D$ −17° 25′ (in 82–83% alcohol).

The structural formula for sinigrin, pictured above, was established by Gadamer.[3]

As regards the ferment myrosinase, Heiduschka and Pyriki[4] carried out experiments in an attempt to prepare a ferment of high potency.

The hydrolysis of the glucoside sinigrin does not always proceed smoothly according to the scheme pictured above, and side reactions may take place. Thus, allyl cyanide and carbon disulfide may be formed. Many years ago Schmidt[5] observed that the allyl isothiocyanate may be accompanied by small quantities of the isomer, allyl thiocyanate, if fermentation is conducted at low temperature. Perhaps allyl thiocyanate is always formed

[1] *Z. physiol. Chem.* **126** (1923), 210.
[2] *J. pharm. chim.* [8], **6** (1927), 337.
[3] *Arch. Pharm.* **235** (1897), 47. *Ber.* **30** (1897), 2322, 2328.
[4] *Arch. Pharm.* **264** (1926), 694.
[5] *Ber.* **10** (1877), 187.

#132

Food Chemicals Codex, First Edition, (1966). Publication 1406. National Academy of Sciences - National Research Council. Washington, D. C. pp ~~[illegible]~~ 24 - 25

ALLYL ISOTHIOCYANATE

Mustard Oil, Volatile

$CH_2{=}CH{-}CH_2{-}N{=}C{=}S$

C_4H_5NCS **Mol. wt. 99.15**

DESCRIPTION

The oil obtained by maceration with water and subsequent distillation of the dried ripe seed (free from fixed oil) of *Brassica nigra* (Linné)Koch, or of *Brassica juncea* (Linné) Czerniaew (Fam. *Cruciferae*), or prepared synthetically. It is a colorless or pale yellow, strongly refractive liquid, having a very pungent, irritating odor and an acrid taste. It is miscible with alcohol, with ether, and with carbon disulfide. It is optically inactive.

Caution: Exercise great care in smelling allyl isothiocyanate. It should be tasted only when highly diluted.

IDENTIFICATION

To a 3-ml. sample, gradually add 3 ml. of sulfuric acid, keeping the mixture cool, then cautiously agitate the liquid. The mixture evolves sulfur dioxide and retains a light yellow color, but loses the pungent odor of the oil.

SPECIFICATIONS

Assay. Not less than 93 per cent of C_4H_5NCS.

Distillation range. Between 148° and 154°.

Refractive index. Between 1.5275 and 1.5310 at 20°.

Specific gravity. Between 1.013 and 1.020.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 per cent).

Heavy metals (as Pb). Not more than 40 parts per million (0.004 per cent).

Lead. Not more than 10 parts per million (0.001 per cent).

Phenols. Passes test.

TESTS

Assay. Transfer about 4 ml., accurately weighed, into a 100-ml. volumetric flask, and add sufficient alcohol to make 100.0 ml. Pipet 5

ml. of this solution into a 100-
N silver nitrate and 5 ml.
a reflux condenser, heat it o
liquid to cool to room temper
denser, add sufficient water t
well, and filter through a dry
To 50 ml. of the subsequent
5 ml. of nitric acid and 2 ml. o
the excess silver nitrate with
blank determination, using 5
tion, and make any necessa
nitrate is equivalent to 4.958

Distillation range. Proc
page 737. The sample distill

Refractive index, page 7
fractometer of equal or greate

Specific gravity. Determ

Arsenic. A *Sample Solut*
pounds meets the requiremen

Heavy metals. Prepare
Method II under the *Heavy*
ion (Pb) in the control (*Solut*

Lead. A *Sample Solution*
pounds meets the requiremen
mcg. of lead ion (Pb) in the

Phenols. Dilute 1 ml. o
1 drop of ferric chloride T.S.

Packaging and storage.
place protected from light.

Labeling. Label allyl isoth
synthetically or distilled from
tion.

Functional use in foods.

ALMOND

Bitter Almond

DESCRIPTION

A volatile oil obtained fr
(De Candolle) Focke (Fam. *F*
L.), and other fruit kernels
steam distillation of a wate

n full, tight, preferably glass, tin-
ners in a cool place protected from

ring agent.

IOCYANATE

il, Volatile

H_2—N=C=S

Mol. wt. 99.15

ch water and subsequent distillation
oil) of *Brassica nigra* (Linné)Koch,
aew (Fam. *Cruciferae*), or prepared
le yellow, strongly refractive liquid,
or and an acrid taste. It is miscible
carbon disulfide. It is optically in-

elling allyl isothiocyanate. It should

3 m.. of sulfuric acid, keeping the
e the liquid. The mixture evolves
yellow color, but loses the pungent

of C_3H_5NCS.
° and 154°.
5 and 1.5310 at 20°.
and 1.020.

3 parts per million (0.0003 per cent).
ore than 40 parts per million (0.004

per million (0.001 per cent).

accurately weighed, into a 100-ml.
alcohol to make 100.0 ml. Pipet 5

ml. of this solution into a 100-ml. volumetric flask, and add 50.0 ml. of 0.1 *N* silver nitrate and 5 ml. of ammonia T.S. Connect the flask to a reflux condenser, heat it on a water bath for 1 hour, and allow the liquid to cool to room temperature. Disconnect the flask from the condenser, add sufficient water to make the mixture measure 100.0 ml., mix well, and filter through a dry filter, rejecting the first 10 ml. of filtrate. To 50 ml. of the subsequent filtrate, accurately measured, add about 5 ml. of nitric acid and 2 ml. of ferric ammonium sulfate T.S., and titrate the excess silver nitrate with 0.1 *N* ammonium thiocyanate. Perform a blank determination, using 5 ml. of alcohol in place of the sample solution, and make any necessary corrections. Each ml. of 0.1 *N* silver nitrate is equivalent to 4.958 mg. of C_3H_5NCS.

Distillation range. Proceed as directed under *Distillation Range*, page 737. The sample distills completely within the specified range.

Refractive index, page 785. Determine with an Abbé or other refractometer of equal or greater accuracy.

Specific gravity. Determine by any reliable method (see page 4).

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 720.

Heavy metals. Prepare and test a 500-mg. sample as directed in *Method II* under the *Heavy Metals Test*, page 763, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Lead. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Lead Limit Test*, page 772, using 10 mcg. of lead ion (Pb) in the control.

Phenols. Dilute 1 ml. of sample with 5 ml. of alcohol, and add 1 drop of ferric chloride T.S. A blue color is not produced immediately.

Packaging and storage. Store in full, tight containers in a cool place protected from light.

Labeling. Label allyl isothiocyanate to indicate whether it was made synthetically or distilled from one of the plants mentioned in the *Description*.

Functional use in foods. Flavoring agent.

ALMOND OIL, BITTER, FFPA

Bitter Almond Oil Free from Prussic Acid

DESCRIPTION

A volatile oil obtained from *Prunus amygdalus* Batsch var. *amara* (De Candolle) Focke (Fam. *Rosaceae*), apricot kernel (*Prunus armeniaca* L.), and other fruit kernels containing amygdalin. It is prepared by steam distillation of a water-macerated, powdered and pressed cake

#14

FLAVORS AND NONALCOHOLIC BEVERAGES

Allyl Isothiocyanate in Mustard Seed

By DONALD L. ANDERSEN (Food and Drug Administration, 909 First Ave., Seattle, Wash. 98104)

A new GLC method for the determination of allyl isothiocyanate in mustard seed was compared to a method of the Midwest Research Institute and to a combination of the AOAC official method and the proposed method. Twelve collaborators compared the AOAC method and the GLC method, using whole mustard seeds. Each collaborator assayed three seed portions by both methods. The range, standard deviation, and coefficient of variation are less for each seed portion by the proposed than by the official method. The average recovery value of allyl isothiocyanate in the prepared standard solutions is lower, using the proposed GLC procedure, but seed assay values are significantly and consistently higher for each seed portion when compared with the results for the AOAC method. Reports from the collaborators also indicate that the proposed method is rugged, as the GLC column preparation was subjected to many changes. It is recommended that the GLC method be adopted as official first action.

Development of a new, more accurate and precise method for the analysis of allyl isothiocyanate has been underway for several years. The procedure under investigation is a modification of the present official method (1). The fermentation medium was changed, fermentation time was decreased, contents of the distillation receiver were slightly changed, and determination step was changed to GLC.

Various columns were investigated to determine their applicability and to show that the GLC response was due only to the allyl isothiocyanate. Columns investigated included polymer beads, silicones, polyesters, and polyglycols. The silicone-coated column (SE-30) was unsatisfactory for quantitation, but the other three types gave essentially the same quantitative results. With each of the three columns, only one peak was observed after the solvent front, which indicates that this peak is due to a single component. The polyglycol type (Carbowax 4000) was selected because it gave good separation and because a similar column is being used by the AOAC for cosmetic analysis (2). In addition, solvent systems and fermentation time and conditions were studied. Also, it was established by mass spectrometry that allyl isothiocyanate remains intact after boiling in alcohol; it is therefore probable that the GLC peak is due to this intact compound.

METHOD

Grind ca 15 g well mixed whole seed to pass No. 20 sieve. Immediately, weigh accurately 6.0 g into 300 ml conical flask and add 150 ml 5% alcohol. Stopper flask and place in 37° H_2O-bath on magnetic stirre 1.5 hr ±5 min (fermentation step). Distill sample into 100 ml vol. flask contg 20 ml 5% alcohol. Condenser delivery tube must dip below surface of receiver liq. Dil. distillate to vol. and inject portion into GLC fitted with 12′ column of 5% Carbowax 4000 on Fluoropak 80 and flame ionization detector. Approx. conditions: column 145°, detector 200°, injector 160°, and N flow 100 ml/min. Compare peak ht of sample to that of std prepd as follows: Add 30.0 µl allyl isothiocyanate (in 50 µl syringe with ±0.5% accuracy) to 50 ml 10% alcohol in 100 ml vol. flask and shake intermittently until dissolved. Dil. to vol. with H_2O (30.5 mg/100 ml).

Methods Comparison

Initially the following four methods were compared: the proposed method, a procedure developed by the Midwest Research Institute (MRI method) (3), a combination of the determinative step of the official method and the fermentation step of the proposed method, and the present AOAC method (*see* Table 1). The results indicate comparability for the proposed method with the combination method. The present AOAC method gives the lowest results and the MRI, intermediate values. This would suggest that the present

Table 1. Comparison of four methods for determining per cent allyl isothiocyanate in same mustard seed composite

Prop.	AOAC	AOAC and Prop. Ferm.	MRI
0.75	0.59	0.74	0.68
0.72	0.57	0.72	0.67

official method should be modified to include the proposed fermentation step. Laboratory investigations have shown that the increased amount of alcohol increases fermentation yield, and that 1.5 hr is optimum for maximum yield.

The MRI method has several drawbacks: the total sulfur determination is non-specific, the silver sulfide precipitation is not allowed to go to completion, and the addition of yellow mustard flour and boiling of the ground seed produce higher results. Each of these is a potential source of error. The MRI report, however, indicates that they feel that results in excess of those obtained by the present AOAC method are closer to true quantitation.

Collaborative Study

A collaborative study was completed this year with 12 collaborators reporting. Each collaborator was instructed to assay three seed portions by the proposed procedure and by the AOAC method and to determine the recovery by each method. Recoveries constituted the analysis of allyl isothiocyanate standard solution beginning with the distillation step.

The overall results (Table 2) show an average recovery of 95.7% for the official method and 90.8% for the proposed method. The results of the proposed method exceed those of the official method by more than 20% for each seed portion. Range and standard deviation values are slightly lower for each seed portion by the proposed procedure.

Four of the twelve collaborators modified the prescribed GLC column. A separate statistical evaluation (Table 3) was made for those collaborators who did not modify the column (Collaborators 2–9). The results obtained from this smaller group differ only minimally from those of the total group, suggesting that the method allows some latitude. The modifications of the column included the use of 6, 8, and 10′ columns in place of the 12′ column specified. In one case a different molecular weight coating was used and in two cases the support material was changed.

Discussion and Recommendation

Few negative comments were received from the collaborators. Several held that the method of GLC quantitation (peak height, triangulations, etc.) should be specified. Upon checking those results received it was found that peak height measurements would have given satisfactory answers in each case.

Table 2. Results of collaborative study for per cent allyl isothiocyanate in mustard seed

	Analysis of Seed Portion										Recovery from Std Soln	
	A		D		G[a]		H[a]		J			
Coll.	AOAC	Prop.	AOAC	Prop.	AOAC	Prop.	AOAC	Prop.	AOAC	Prop.	AOAC	Prop.
1	0.44	0.70	0.57	0.78							98.5	90.4
2	0.47	0.75	0.64	0.87					0.51	0.87	85.3	84.6
3	0.49	0.68	0.60	0.79					0.58	0.78	92.3	95.1
4	0.56	0.75	0.71	0.82					0.62	0.81	95.2	93.7
5	0.61	0.66	0.77	0.77					0.73	0.82	96.5	85.2
6	0.55	0.64	0.63	0.84					0.62	0.81	96.8	87.7
7	0.51	0.61	0.56	0.79					0.71	0.79		
8	0.52	0.72	0.65	0.92					0.81		95.2	92.5
9	0.45	0.72	0.59	0.77							100.1	99.2
10		0.69		0.88		0.72						84.5
11			0.86	0.86	0.67	0.73	0.69	0.74			96.9	85.7
12			0.76	0.95	0.62	1.01		0.93			100.0	100.7
Range	0.17	0.14	0.30	0.18					0.30	0.09	14.8	16.2
Av.	0.51	0.69	0.67	0.84					0.65	0.81	95.7	90.8
Std dev.	0.056	0.046	0.095	0.060					0.10	0.03	4.34	5.86
Coeff. of var.	11.0	6.7	14.2	7.1					15.4	4.0	4.54	6.45

[a] Portions G and H are identical. Insufficient data were obtained to draw any statistical conclusion about portions G and H.

Table 3. Statistical summary of results of collaborative study from eight laboratories[a]

Statistic	Seed Portions[b]			
	A	D	J	Rec.
Range, %	0.14	0.15	0.09	14.6
Av., %	0.69	0.82	0.81	91.1
Std dev.	0.052	0.053	0.032	5.46
Coeff. of var.	7.5	6.5	4.0	6.0

[a] These eight collaborators did not modify gas chromatographic column specified in method.
[b] These collaborators did not work on portions G or H.

The proposed method is more specific, shows slightly better range, standard deviation, and coefficient of variation values, and produces consistently and significantly higher results.

It is recommended that the proposed procedure be adopted as official first action for the determination of allyl isothiocyanate in mustard seed.

This report of the Associate Referee was presented at the 83rd Annual Meeting of the AOAC, Oct. 13–16, 1969, at Washington, D.C.

Acknowledgments

The Associate Referee would like to thank the following persons for their assistance in this study: J. Burkel, Dallas; J. Jones, Boston; R. Thompson, Minneapolis; J. Stilz, Cincinnati; J. Harrell, Atlanta; R. Frank, Baltimore; A. Taira, Chicago; Dalia Gilvydis, Detroit; J. Fetters, Denver; and W. Saxton, Seattle (all of the Food and Drug Administration); and W. Chilton, University of Washington, Seattle; P. Guarino, McCormick & Co., Baltimore, Md.; and J. DiCecco, R. T. French Co., Rochester, N.Y.

References

(1) *Official Methods of Analysis*, 10th Ed., AOAC, Washington, D.C., 1965, sec. 28.023.
(2) "Changes in Methods," *JAOAC* **49**, 242–244 (1966).
(3) Rosebrook, D. D., and Barney, J. E., II, *JAOAC* **51**, 633–636 (1968).

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee D and was adopted by the Association; their reports will appear in *JAOAC* **53**, March 1970.

Specifications of Fatty Acid Composition for Identification of Fats and Oils by Gas Liquid Chromatography

ROBERT T. O'CONNOR, Southern Regional Research Laboratory,[1] New Orleans, Louisiana 70119 and S. F. HERB, Eastern Regional Research Laboratory,[2] Philadelphia, Pennsylvania 19118

The Codex Committee on Fats and Oils of the Joint Food and Agricultural Organization of the United Nations and the World Health Organization (FAO/WHO), Codex Alimentarius Commission, has for several years had under consideration a complete revision of their standards for the identification of fats and oils. As early as 1966, the Canadian Delegation proposed that the classical method be replaced by an alternate system based on the fatty acid composition of each fat or oil as determined from gas liquid chromatographic patterns. The classical method is based on the identification of a specific fat or oil by means of a combination of its iodine value, relative density, refractive index and saponification value.

Prior to the third meeting of the Fats and Oil Committee held in London April 24–28, 1967, the Canadian Delegation submitted a proposal for the replacement of the classical method with a gas liquid chromatographic (GLC) procedure. In this proposal the Canadian Delegation discussed disadvantages of the classical system, particularly the requirement for a heavy investment of analytical time and skill in many techniques, and inability, even when all four indices are considered, to resolve several admixtures containing common oils of commerce. At the same time, advantages of the gas liquid chromatographic procedure were emphasized, including the fact that it permits identification of oils which cannot be identified by the classical method and offers promise of resolving many additional pairs of oils in admixture. Furthermore, since it requires only one analysis, it can be made quite rapidly and applies equally well to refined and unrefined oils, necessitating only one set of standards.

From the beginning, at least as early as 1966, the United States supported the Canadian proposal in principle and recommended specifically that the Codex Committee on Fats and Oils urge the Codex Committee on Methods of Analysis to evaluate the current status of the science of gas liquid chromatography and, if feasible, to adopt GLC procedures that can be used by the Codex Committee on Fats and Oils.

At the fourth meeting of the Codex Committee on Fats and Oils, sponsored by the Joint FAO/WHO Codex Alimentarius Commission and held in London, April 24–28, 1967, the Canadian proposal was presented. The U.S. position on the proposal was one of partial support. This position was explained: "Full support of the Canadian proposal would not be justified, since most available evidence indicates that adoption of the proposal at this time would be premature and would lead to serious practical difficulties. Full opposition to the proposal would place the United States in a position of being ultra-conservative and unwilling to recognize the most modern scientific advances in the field of fat and oil analysis. The U.S. delegation therefore took the position that a system of identification based on GLC, in addition to the classical system, be carried in the draft standards at this stage. This would be done with the expectation that before final promulgation of the standards, the science of GLC will have progressed to a point where the present uncertainties concerning it would be eliminated and that it would provide a far more accurate means of identification than is possible with the present classical procedures. If this expectation should be realized, the classical system could then be dropped before final promulgation. The U.S. delegation argued that acceptance of its views would encourage the necessary international cooperation needed in the further perfection of the GLC system of oil and fat identification." After considerable discussion this U.S. position prevailed among the delegates from the 21 nations represented at this fourth meeting.

In comments on the Canadian proposal, submitted to delegates of the countries to be represented prior to the April 1967 Meeting, the United States delegation had presented its position, as given above. With their comments the delegation commented on the requirement for GLC standards and proposed to solicit recommendations concerning such standards from the Codex Committee on Methods of Analysis which would likely be based on the work of the International Union of Pure and Applied Chemistry. In these remarks they commented that the American Oil Chemists' Society, through its Instrumental Techniques Committee and the Gas Chromatography Subcommittee thereof, was currently very active in this field and "could probably render valuable assistance to the Codex Committee on Methods of Analysis" hopefully prior to the April 1967 meeting.

The hope for accomplishment of this objective prior to the April 1967 meeting was apparently not realized, but shortly after acceptance of its proposal at this meeting, the United States delegation, through its Chairman Lawrence Zeleny, contacted the Society with a request for assistance in the preparation of such standards. The AOCS Executive Director, C. H. Hauber, arranged a meeting which was held during the joint AACC-AOCS 59th Annual Con-

(Continued on page 195A)

[1] So. Utiliz. Res. Dev. Div., USDA.
[2] E. Utiliz. Res. Dev. Div., USDA.

TABLE I

Arachis Oil[a,b]

Acids	Carbon atoms	Range
Saturated	Less than 14	<0.5
Myristic	14:0	<1.0
Palmitic	16:0	6.0–14
Stearic	18:0	2.0– 6.5
Arachidic	20:0	1.0– 2.0
Behenic	22:0	2.0– 4.0
Lignoceric	24:0	1.0– 2.0
Palmitoleic	16:1	<1.0
Oleic	18:1	40 –72
Linoleic	18:2	13 –38
Linolenic	18:3	<0.50
Eicosenoic	20:1	0.5– 1.5

[a] Synonyms: peanut, groundnut.
[b] Fatty acid composition by gas chromatography (wt % of methyl esters).

TABLE II

Cottonseed Oil[a,b]

Acids	Carbon atoms	Range
Saturated	Less than 14	<0.5
Myristic	14:0	0.5– 2.0
Palmitic	16:0	17 –29
Stearic	18:0	1.0– 4.0
Arachidic	20:0	<0.5
Behenic	22:0	....
Lignoceric	24:0	....
Palmitoleic	16:1	<1.5
Oleic	18:1	13 –44
Linoleic	18:2	33 –58
Linolenic	18:3	<0.5
Eicosenoic	20:1	<0.5

[a] Synonyms: none.
[b] Fatty acid composition by gas chromatography (wt % of methyl esters).

(Continued from page 186A)

TABLE III
Maize Oil[a,b]

Acids	Carbon atoms	Range
Saturated	Less than 14	<0.5
Myristic	14:0	<1.0
Palmitic	16:0	8 –19
Stearic	18:0	0.5– 4.0
Arachidic	20:0	<1.0
Behenic	22:0	....
Lignoceric	24:0	....
Palmitoleic	16:1	<1.0
Oleic	18:1	19 –50
Linoleic	18:2	34 –62
Linolenic	18:3	<1.0
Eicosenoic	20:1	<0.5

[a] Synonyms: corn.
[b] Fatty acid composition by gas chromatography (wt % of methyl esters).

TABLE IV
Mustardseed Oil[a,b]

Acids	Carbon atoms	Range
Saturated	Less than 14	<0.5
Myristic	14:0	<1.0
Palmitic	16:0	0.5– 4.5
Stearic	18:0	0.5– 3.0
Arachidic	20:0	<1.5
Behenic	22:0	0.2– 2.5
Lignoceric	24:0	<0.5
Palmitoleic	16:1	<1.0
Oleic	18:1	8.0–23.0
Linoleic	18:2	10.0–24.0
Linolenic	18:3	6.0–18.0
Eicosenoic	20:1	4.0–13.0
Erucic	22:1	22.0–49.0
Docosadienoic	22:2	<1.0
Tetracosenoic	24:1	0.5– 2.5

[a] Synonyms: none.
[b] Fatty acid composition by gas chromatography (wt % of methyl esters).

TABLE V
Rapeseed Oil[a,b]

Acids	Carbon atoms	Range
Saturated	Less than 14	<0.5
Myristic	14:0	<1.0
Palmitic	16:0	0.5– 5.0
Stearic	18:0	0.5– 3.0
Arachidic	20:0	<1.5
Behenic	22:0	<1.5
Lignoceric	24:0	<2.0
Palmitoleic	16:1	<1.0
Oleic	18:1	9.0–25
Linoleic	18:2	11 –25
Linolenic	18:3	5.0–12
Eicosenoic	20:1	5.0–15
Erucic	22:1	30 –60
Docosadienoic	22:2	<1.0

[a] Synonyms: colza, ravison, sarson, toria, turnip rape.
[b] Fatty acid composition by gas chromatography (wt % of methyl esters).

TABLE VI
Safflower Seed Oil[a,b]

Acids	Carbon atoms	Range
Saturated	Less than 14	<0.5
Myristic	14:0	<1.0
Palmitic	16:0	2.0–10
Stearic	18:0	1.0– 6.0
Arachidic	20:0	<1.0
Behenic	22:0	<1.0
Lignoceric	24:0	....
Palmitoleic	16:1	<0.5
Oleic	18:1	7.0–40
Linoleic	18:2	55 –80
Linolenic	18:3	<1.0
Eicosenoic	20:1	<0.5

[a] Synonyms: carthamus, durdee.
[b] Fatty acid composition by gas chromatography (wt % of methyl esters).

vention in the Washington-Hilton Hotel, Washington, D.C., Tuesday, April 2, 1968. The minutes of this meeting show that it was attended by five United States or Canadian delegates or advisors to the delegates to the Codex Committee on Fats and Oils, by four members of the American Oil Chemists' Society, and by two members from American industrial organizations.

At this meeting the requirements of the Codex Committee were set forth by the U.S. Delegates. This delegation had felt in the past that the proposal to use GLC in Codex standards had been premature because of insufficient knowledge of the ranges in fatty acid composition characteristics of some of the fats and oils. However recent developments in this field had led them to the opinion that Codex standards should utilize GLC analysis at least on an optional basis, otherwise standards based entirely on the classical fat and oil characteristics would soon become obsolete.

It was emphasized by the AOCS members that the major problem in establishing such standards is the need for a comprehensive survey of the variability in fatty acid distribution for each product for which standards are to be developed in order to establish completely valid and at the same time reasonable minimum and maximum requirements for such standards. It was agreed that the Codex delegation would furnish a list of the fats and oils for which standards were now required and that the AOCS, through its Instrumental Techniques Committee, would initiate the necessary survey to establish the minimum and maximum limits for each fatty acid in each specific fat or oil.

It was also agreed that in establishing these standards, the range for each fatty acid in each fat or oil would be broad enough to include all products ordinarily encountered in world trade, the complete range expected for commercial fats or oils, but not necessarily broad enough to include all products which might be encountered under exceptional conditions. Exceptional oils, it was pointed out, can result from agronomic or genetic advances to impart special properties to a specific oil. Three examples are given to illustrate such exceptional oils (where the minimum or maximum concentration or both of one or more acids might be outside of the normal range for oils of commerce.)

1. Safflower oil can be of high linoleic or high oleic variety. In the former, the 18:2 acid would be 55% to 80% and 18:1 7.0% to 40%; in the latter, the 18:2 would be 7.0% to 40% and 18:1 55% to 80%. To cover both, 18:1 would be 7.0% to 80% and 18:2 also 7.0% to 80%.
2. A rapeseed oil has now been developed which contains no erucic acid (22:1) so if this unusual variety was included, this component would be 0% to 60%, 22:1 instead of 50% to 60%.
3. The fatty acid composition for arachis oil has been found to be "unusual" in the following cited examples: Arachidic was found to be less than 1% in eight of 11 varieties studied (1). Lignoceric was found to be less than 1% in one out of three samples investigated by Craig and Murthy; for one out of seven varieties (2), and one out of 22 samples (3).

The delegates to the Codex Committee on Fats and Oils requested specifications for the following fats and oils: arachis oil (peanut oil) (groundnut oil), cottonseed oil, maize oil (corn oil), rapeseed oil, safflower oil, sesame seed oil, soya bean oil (soybean oil), sunflower seed oil, lard (rendered pork fat), and premier jus (edible tallow). Mustard seed oil was added to this list after the fifth meeting of the Codex Committee on Fats and Oils in London, September 16–20, 1968.

The Chairman of the Gas Chromatography Subcommittee of the Instrumental Techniques Committee prepared a list, from an exhaustive search of the literature, of the minimum-maximum range of fatty acid composition, as obtained by gas chromatography. These values were submitted to several

(Continued on page 197A)

(Continued from page 195A)

TABLE VII
Sesame Seed Oil[a,b]

Acids	Carbon atoms	Range
Saturated	Less than 14	<0.5
Myristic	14:0	<0.5
Palmitic	16:0	7.0–12
Stearic	18:0	3.5– 6.0
Arachidic	20:0	<1.0
Behenic	22:0	<1.0
Lignoceric	24:0	....
Palmitoleic	16:1	<0.5
Oleic	18:1	35 –50
Linoleic	18:2	35 –50
Linolenic	18:3	<1.0
Eicosenoic	20:1	<0.5

[a] Synonym: gingelly.
[b] Fatty acid composition by gas chromatography (wt % of methyl esters).

TABLE VIII
Soya Bean Oil[a,b]

Acids	Carbon atoms	Range
Saturated	Less than 14	<0.5
Myristic	14:0	<0.5
Palmitic	16:0	7.0–12
Stearic	18:0	2.0– 5.5
Arachidic	20:0	<1.0
Behenic	22:0	<0.5
Lignoceric	24:0	....
Palmitoleic	16:1	<0.5
Oleic	18:1	20 –50
Linoleic	18:2	35 –60
Linolenic	18:3	2.0–13
Eicosenoic	20:1	<1.0

[a] Synonym: soybean.
[b] Fatty acid composition by gas chromatography (wt % of methyl esters).

TABLE IX
Sunflower Seed Oil[a,b]

Acids	Carbon atoms	Range
Saturated	Less than 14	<0.5
Myristic	14:0	<0.5
Palmitic	16:0	4.0– 9.0
Stearic	18:0	1.0– 6.0
Arachidic	20:0	<1.0
Behenic	22:0	<1.0
Lignoceric	24:0	....
Palmitoleic	16:1	<0.5
Oleic	18:1	14 –70
Linoleic	18:2	20 –75
Linolenic	18:3	<0.5
Eicosenoic	20:1	<0.5

[a] Synonym: none.
[b] Fatty acid composition by gas chromatography (wt % of methyl esters).

TABLE X
Lard (and Rendered Pork Fat)[a,b]

Acids	Carbon atoms	Range
Saturated	Less than 14	<1.0
Myristic	14:0	0.5– 2.5
Palmitic	16:0	20 –32
Stearic	18:0	5.0–18
Arachidic	20:0	<1.0
Behenic	22:0	<0.5
Lignoceric	24:0	....
Palmitoleic	16:1	2.0– 4.0
Oleic	18:1	35 –60
Linoleic	18:2	3.0–15
Linolenic	18:3	<2.0
Eicosenoic	20:1	<1.0

[a] Synonym: none.
[b] Fatty acid composition by gas chromatography (wt % of methyl esters).

TABLE XI
Premier Jus (and Edible Tallow)[a,b]

Acids	Carbon atoms	Range
Saturated	Less than 14	<1.0
Myristic	14:0	1.0– 8.0
Palmitic	16:0	23 –37
Stearic	18:0	6.0–20
Arachidic	20:0	<1.0
Behenic	22:0	....
Lignoceric	24:0	....
Palmitoleic	16:1	1.5– 6.0
Oleic	18:1	36 –50
Linoleic	18:2	0.5– 5.0
Linolenic	18:3	<1.0
Eicosenoic	20:1	<1.0

[a] Synonyms: none.
[b] Fatty acid composition by gas chromatography (wt % of methyl esters).

members of AOCS knowledgeable in fatty acid composition of fats and oils and from their remarks, comments and suggestions final ranges were selected as GLC specifications.

These specifications were submitted to the U.S. Delegation to the Codex Committee on Fats and Oils, through their Chairman, Dr. Zeleny, and presented at the fifth session of the Codex Committee on Fats and Oils, sponsored by the Joint FAO/WHO Codex Alimentarius Commission and held in London September 16–20, 1968. The specifications were, according to the minutes of this meeting, well received. However, as the delegates had not had an opportunity to study them, the United States proposal ". . . that all of the Codex standards for specified kinds of fats and oils contain two alternative sets of identification requirements, one based on the classical physical and chemical characteristics and the other on fatty acids composition as determined by GLC," while supported by a majority of the delegates, was referred by the Chairman of the Fifth Session back to the delegates with the request for ". . . all participating countries to study the U.S. proposal further and to consider at the next meeting of the Committee whether GLC procedures for identifying fats and oils should be inserted into the standards as 'advisory criteria.'"

At this Fifth Session of the Codex Committee on Fats and Oils, standards for mustard seed oil were discussed. One outcome of this discussion was a request to the AOCS for inclusion of standards for this oilseed, from GLC measurements. These were subsequently furnished to M. H. Neustadt, who succeeded Lawrence Zeleny as Chairman of the U.S. Delegation.

In the meantime considerable interest has been made evident by several members of AOCS, usually with requests for the GLC specifications. It appears that the most suitable medium to make the specifications available to any interested member of the Society, while they are being considered by the Codex Committee on Fats and Oils, would be by their publication in the *Journal*.

Complete specifications for the 11 oilseeds thus far considered, including mustard seed oil, are given in the attached Tables.

REFERENCES

1. French, R. V., JAOCS *39*, 176 (1962).
2. Worthington, R. E., and K. T. Holley, JAOCS *44*, 515 (1967).
3. Iverson, J. L., D. Firestone and W. Horwitz, J. Assoc. Offic. Anal. Chemists *46*, 718 (1963).

[Received December 22, 1969]

Fatty Acids Statistics

Production of animal, vegetable, and marine fatty acids totalled 51.7 million pounds in January 1970. Inclusion of the tall oil types raised the overall January production level to 84.2 million pounds.

Disposition of fatty acids amounted to 64.7 million pounds in January. Including tall oil fatty acids, January 1970 disposition totalled 97.6 million pounds.

Stocks of fatty acids other than the tall oil types amounted to 39.7 million pounds on January 31st, down 7.7 million pounds from January 1st.

Source: Fatty Acid Producers' Council, 485 Madison Ave., New York, N.Y. 10022.

MICRO-ESTIMATION OF MAJOR MUSTARD OILS AND OXAZOLIDINETHIONE IN SMALL AMOUNTS OF PLANT MATERIAL

By P. LANGER and K. GSCHWENDTOVÁ

A method for the separate measurement of volatile and non-volatile mustard oils in small amounts of plant material is described. Thioglucosides are first preserved by boiling the plant material in alcohol. After evaporation of the alcohol and enzymic splitting of thioglucosides the aglucones are extracted with ether. From this extract volatile mustard oils may be easily evaporated together with ether, and the non-volatile mustard oils remain in the residue. By this method mustard oils were fractionated into ether-volatile and ether-non-volatile fractions. Both of them are quantitatively measured with the aid of ultra-violet spectrophotometry in the range 230–260 nm. Separation of the individual compounds from these fractions by paper chromatography is described, and qualitative and quantitative aspects of this method are discussed.

Introduction

Estimation of mustard oils by standard methods was reviewed by André & Maille[1] and Stoll & Jucker.[2] One of the most commonly used methods was that of Gadamer[3] in various modifications, from which that of Schuphan[4] and in recent times that of Wetter[5] were based. The common principle of these methods is the transformation of the mustard oils into monosubstituted thioureas, from which the insoluble silver sulphide and monosubstituted cyanamide are formed through the interaction with ammoniacal silver nitrate. Finally, the silver residue is measured volumetrically.

Mustard oils were originally separated from natural material with the aid of steam distillation; recently extraction by various organic solvents has been used.[6] Recent methods have provided a separation either of the individual naturally occurring isothiocyanates, following their transformation into thiourea derivatives, with the aid of paper chromatography;[6] or of the individual isothiocyanates directly, with the aid of gas chromatography.[7,8] Compounds isolated by extraction or chromatography, or by a combination of the two, are finally measured by ultra-violet spectrophotometry.[9–12] All these newer methods were used in some preparative studies of the thioglucosides, particularly mustard oils, and other aglucones arising from them in various plants or seeds.[13–17,18] With some exceptions[5,10–12] predominantly qualitative studies were made. Reviews have been published by Kjaer[17] and Gmelin[19] and the quantitative estimation in rape seeds was studied by Appelquist & Josefsson[20] and even on a micro-scale by Youngs & Wetter.[8] The aim of this paper is to present results from extensive experience of the estimation of mustard oils in plant material, used in some studies of the antithyroidal effect of these compounds.[21,22]

Experimental

Reagents

Peroxide-free ether, 0·2 M phosphate buffer, pH 7·0 (according to Sörensen), 0·005 M ascorbic acid in distilled water (freshly prepared), alcohol (ethanol or methanol), ammonium hydroxide conc., (analytical grade), myrosinase prepared from white mustard seeds (*Sinapis alba*).[23]

Reference substances: allylisothiocyanate (Lachema, Czechoslovakia) and allylthiourea (Light, England) and other naturally occurring isothiocyanates, or corresponding thioureas, were used.

Distillation apparatus (Fig. 1).

Procedure

Preparation of the extract from the plant material

Weighed leaves (1–10 g) were immersed in 5–10 times their weight of boiling alcohol. After 5 minutes of boiling, the mixture was cooled and transferred into a Sorvale Omni-mixer and homogenised. It was then filtered through a Buchner funnel into a previously weighed round-bottomed boiling flask. The tissue remaining in the funnel was washed 2–3 times with 10–20 ml of hot alcohol. The filtrate was then evaporated under reduced pressure at 45–50°, until the weight of the extract decreased to ~ 10% of the original weight of the plant material.

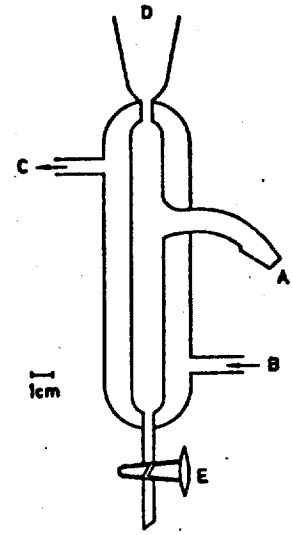


FIG. 1. *Scheme of the distillation apparatus*
A, joint for the round bottom flask, containing the ether extract; B, C, water flow; D, joint for the reflux; E, stopcock

Enzymic splitting and ether extraction

0·05–0·20 ml of the plant extract was measured into a test tube and then phosphate buffer was added to a final volume of 0·50 ml. This mixture was extracted 3 times with 3 ml of ether. The combined ether extract (first extract) contained most of the ether-extractable interfering substances (Fig. 2).

After the first extraction 0·10 ml of the ascorbic acid solution and 0·10 ml of myrosinase were added to the residue. This was incubated for 2 hours at room temperature.

After incubation, the mixture was extracted 3 times with 2 ml aliquots of ether. The combined ether extracts (second extract) contain isothiocyanates (ITC) and L-5-vinyl-2-thiooxazolidone (VTO) formed by the enzymic splitting of the thioglucosides. Thus, all the ether-extractable substances from the reaction mixture could be divided into these two ether extracts (Fig. 2).

Quantitative estimation of the total ether-volatile and ether-non-volatile mustard oils (or aglucones)

Two samples of the second ether extract were necessary. To the first 0·5 ml of ammonium hydroxide was added, and the mixture was incubated for 16–24 hours at room temperature with occasional shaking, and was then evaporated to dryness under reduced pressure. In this manner all mustard oils present were transformed into the corresponding monosubstituted thioureas. The residue was then dissolved in 6 ml of ether and measured in a 1 cm stoppered cell at 240–260 nm. Maximum absorption usually occurred at 250 nm. The average of the absorbances at 240 and 260 nm was subtracted from the maximum absorbance.

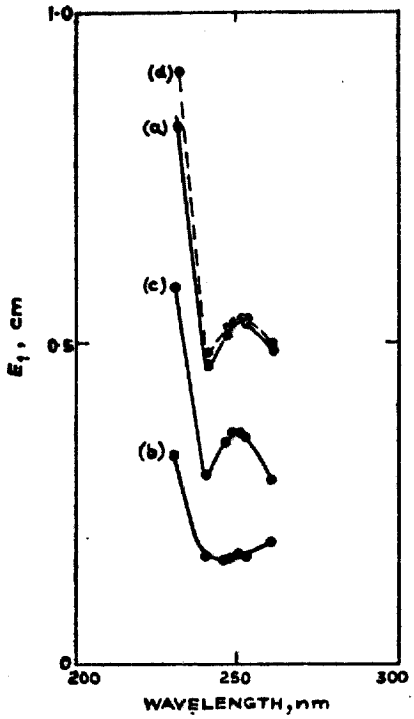


FIG. 2. *U.v. absorption spectra of various extracts from cabbage*
(a) total extract (extraction made after incubation with myrosinase without first extraction); (b) first extract; (c) second extract; (d) theoretical sum of the curves (b) and (c)

The second sample was evaporated to dryness with an infra-red lamp, 6 ml of ether were added once or twice and in each case evaporated. The residue was dissolved in 6 ml of ether and measured as mentioned above. Maximum absorption usually occurred at 248 nm. With the aid of this repeated evaporation all volatile mustard oils were removed while the non-volatile compounds remained, such as VTO, methyl-sulphinyl-butyl-ITC (sulphoraphan) and one unidentified substance. The average values of the absorbances at 236 and 260 nm were subtracted from the absorbances of the total compounds (first sample). The concentration of allylthiourea was estimated from the corrected extinction by comparison with a standard calibration curve. The content of total ether-volatile mustard oils was then expressed, following a stoicheiometric calculation, as the content of allylisothiocyanate (AITC) in 100 g of the original material.

The final extinction of the second sample was compared to a calibration curve of VTO in ether, and the total content of the ether-non-volatile compounds was expressed as the content of VTO in 100 g of the original material.

Estimation of the individual ether-volatile mustard oils by distillation and paper chromatography

The second extract was used. This may be prepared from larger amounts of the plant extract, if necessary, with the aid of correspondingly larger volumes of reagents.

The flask containing the second extract was attached to the distillation apparatus, and evaporated to dryness with the infra-red lamp. The distillate was condensed and after 1–2 minutes was released into a round bottom flask containing concentrated ammonium hydroxide to give a final volume approximately 10% that of the collected distillate. After 16–24 h this mixture was evaporated to dryness and the residue was washed 3–5 times with hot alcohol into a test tube.

Aliquot volumes of this solution were applied on Whatman No. 1 chromatographic paper. The chromatogram was developed in a glass tank, in a descending system, with chloroform saturated with water. After detection the spots were cut out, and extracted twice with 3 ml of hot alcohol for 5–10 min, in a boiling water bath. The extracts are measured against alcohol at 230–260 nm. The average absorbance of each extract at 230 and 256 nm was subtracted from the maximum which usually occurred at 243 nm. The sum of the resulting absorbances was considered as 100%. The percentage representation of the individual compounds in the second extract could then be calculated.

Estimation of the individual ether-non-volatile compounds

The residue after evaporation of the second extract to dryness (see above) was dissolved in ether, and ~ 1/10 volume ammonia was added. The same procedure was then followed as described above.

Results

Preparation of the extract from the plant material

Michajlovskij (personal communication) after boiling crystalline sinigrin for 5 minutes in alcohol found losses of about 10%. From this it may be supposed that some losses may also occur during the boiling of the plant material with alcohol. Yields were lower after extraction of the plant material with boiling alcohol[19] than with the extraction and homogenisation used in this study. After evaporation of the alcohol under reduced pressure at 40–60° the yields were the same.

Estimation

Some ether-extractable substances were present in the myrosinase used. One of them was toluol; others were mustard oils, the presence of which was proved by the following procedure.

Within 30 minutes maximum cleavage occurred; the concentration of ITC did not increase further up to 120 minutes. Yields were the same after 120 minutes at 20–40° or at pH from 6·0–9·0 irrespective of the buffer solution used.

The extraction of 80–90% of the compounds with a maximum absorption at 248 nm (mustard oils and VTO) was repeatedly observed.

Reaction of isothiocyanates with ammonia in the ethereal medium under the conditions described above proceeded quantitatively (Table 1).

Samples evaporated to dryness were dissolved in ether immediately before spectrophotometry. The absorbance of samples in the closed cell was not changed even after 3 hours.

Ultra-violet absorption spectra analysis

The maximum absorption of the second extract was at 248 nm only after the addition of myrosinase to the reaction mixture (Fig. 3). Thus, compounds under observation were specific products of thioglucoside hydrolysis. Both the wavelength and form of the absorption curve either of the second extract or the AITC dissolved in ether were almost identical. AITC, however, may be evaporated almost completely with the ether. On the other hand only a slight decrease in the maximum absorption of the second extract was observed after evaporation and solution in the same volume of ether as present before. However, AITC added to the second extract was also evaporated almost quantitatively. Thus, the second extract contained at least two fractions of compounds showing the same maximum absorption in ether. One of them was ether-volatile. When increasing volumes of plant extract were used, a linear increase of both fractions was observed.

The reaction of both fractions with ammonia was investigated. If 6 ml of ether and 0·5 ml of ammonia were added after the evaporation of the second extract to dryness, the solution was evaporated for 16–24 hours and the residue was dissolved in 6 ml of ether, the absorption spectrum remained unchanged. If the absorption spectrum of the volatile fraction was measured, only a low peak at 248 nm was usually found. After the addition of 0·5 ml of ammonia to this sample, then incubation and evaporation to dryness, and if the solution was in 6 ml of ether, a peak was observed at 250 nm, many times higher than before. This increase resulted from the higher molar extinction coefficient of monosubstituted thioureas compared with that of the corresponding ITC. From this it may be concluded that the reaction of the volatile fraction with the ammonia is different from that of the non-volatile fraction. That the compounds in the volatile fraction are changed into thioureas is demonstrated by the agreement of the ultra-violet absorption spectrum of this fraction with the spectrum of allylthiourea in ether (Fig. 4). Reaction with the ammonia, from which thiourea formation results, is one of the most typical reactions of ITC.[1,2] Therefore it is concluded that ITC is present in this fraction, but not in the non-volatile fraction.

The maximum absorption of the non-volatile fraction was the same as that of VTO in ether (Fig. 4).

On the basis of the ultra-violet absorption spectra, the ether-volatile fraction contained mostly mustard oils, which according to present knowledge are steam-volatile, while the ether-non-volatile fraction contains mostly VTO, which is not volatile in steam.[19]

Paper chromatography

Qualitative composition of the individual fractions of the ether extract was investigated in many cabbages. The maximum number of the compounds observed were, however, not found in every cabbage.

The first extract contained some fluorescent compounds. Most of these were found at the origin, or just behind the solvent front. Part of them originated from the paper and from the solvents used. It could not be completely removed. In the second extract (after the reaction with ammonia) the most pronounced spots were VTO (R_f=0·92) and AITC

TABLE I

Conversion of allylisothiocyanate into allylthiourea

Amount of AITC used, µg	Amount of allylthiourea, µg	
	Calculated	Found
100	117	125
150	175	172
200	234	236

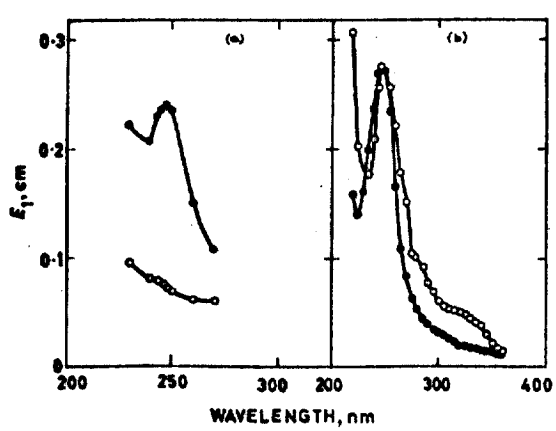


FIG. 3. (a) *U.v. absorption of the first extract* (○) *and the second extract* (●) *and* (b) *of AITC in ether* (●) *and the second extract* (○)

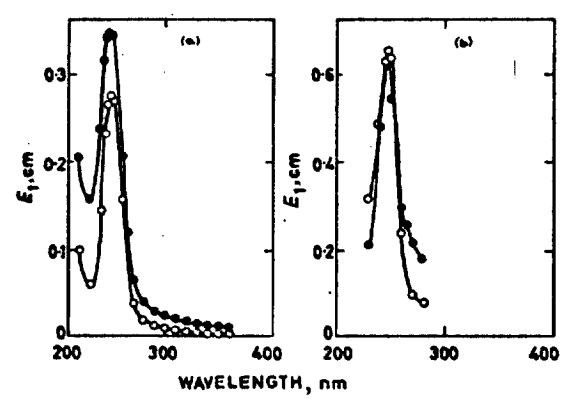


FIG. 4. (*a*) *Relationship between the u.v. absorption spectrum of the second extract after conversion into thioureas* (○) *and allylthiourea* (●) *and* (*b*) *relationship between the u.v. absorption spectrum of VTO in ether* (○) *and the residue of the second extract* (●)

($R_f = 0 \cdot 34$). The latter was present in all samples. The residue after evaporation of the second extract was largely VTO. In addition, an unidentified compound ($R_f = 0 \cdot 85$) usually occurred. Part may be evaporated and thereafter occurs in the volatile fraction also. In the second extract, and in the residue, a spot sometimes occurs of the sulphoraphan (methyl-sulphinyl-butyl-ITC), a small part of which may be observed in the distillate also ($R_f = 0 \cdot 04$).

Qualitative composition of the distillate is presented in Fig. 5. Presumably an almost pure mixture of naturally occurring isothiocyanates was present, containing a small amount of interfering substances only. This condition is of decisive importance for the elution of spots and the quantitative estimation of the individual compounds. In the distillate the following isothiocyanates were observed (in the form of corresponding thioureas): allylisothiocyanate ($R_f = 0 \cdot 34$), 3-butenyl-isothiocyanate ($R_f = 0 \cdot 63$), n-butyl-isothiocyanate ($R_f = 0 \cdot 80$) and two unidentified compounds. One of them had an $R_f = 0 \cdot 71$. On the basis of some reports[13–15] it was considered to be methyl-thio-propyl-ITC. It was not possible, however, to prove an identity of the R_f value of this compound with an authentic sample of the compound mentioned above. The second unknown is very probably identical with a compound usually present in a greater concentration in the residue ($R_f = 0 \cdot 85$).

Ether-volatile ITC was very easily estimated spectrophotometrically after elution. However, only in some cases was sulphoraphan definitely detected after elution. It was situated in the zone of interfering compounds near the origin, which probably made its estimation difficult. Maximum absorption in spectrophotometrically positive cases was at 248 nm. Estimation of VTO was also frequently difficult because it occupied the same region as interfering substances on the chromatogram. Maximum absorption was at 248 nm. An unidentified compound having an R_f value of 0·85 had maximum absorption at 248 nm, and was spectrophotometrically demonstrable in almost all cases, because it was situated in an area free from interfering zones.

The qualitative composition of the individual fractions of the ether extract was thus confirmed by paper chromatography.

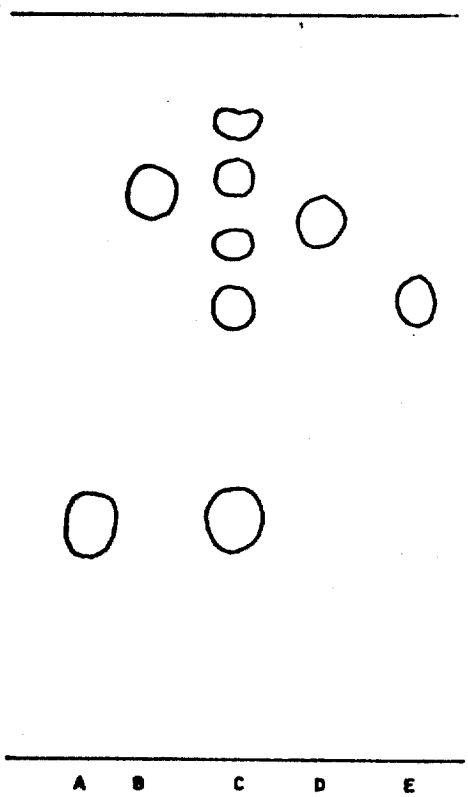


FIG. 5. *Trace of the chromatogram in u.v. light*
A, allylthiourea; B, n-butylthiourea; C, distillate of the second extract; D, methylthio-propylthiourea; E, 3-butenylthiourea

Control of the quantitative aspects of the method

Some data demonstrating that no important losses of the substances under estimation occurred were presented above. Incomplete enzymic splitting of thioglucosides may be a cause of further losses. In this case the specificity of myrosinase[14] and also other factors[12] might be of importance. However, no special studies of them have been made. After the determination of the optimum conditions of enzymic splitting similar methods were used as in other studies.[19] Further losses may result from the ether extraction, which gives yields of 80–90% of the added AITC (Table II), and from the extreme volatility of some isothiocyanates. Thus the final results are probably somewhat lower than the true content in the plant material of the compounds investigated. However, no factors were observed which might artificially increase the concentration of these compounds.

The sensitivity and the accuracy of the estimation is highest when isothiocyanates are converted into thioureas which show much higher molar extinction.

During distillation, losses of the volatile isothiocyanates were constantly about 25% (Fig. 6). The cause of this remains to be elucidated.

By the use of about 10 parallel samples, it was demonstrated that the standard deviation of this procedure was within ± 5% of the average value (Table II). Recovery experiments with the added thioglucosides have not been done because of a lack of the authentic substances.

Discussion

The present method of quantitative determination of mustard oils employs a preliminary extraction of the interfering substances before myrosinase addition, and removal of almost all volatile mustard oils from the ether extract by distillation, to obtain a mixture of naturally occurring mustard oils of relatively high purity. These findings may be of theoretical importance, since they indicate that isothiocyanates and organic solvents may form azeotropic mixtures.

TABLE II

Standard deviation of the measurement of volatile mustard oils in cabbage, and recovery of added allylisothiocyanate

Experiment	No. of parallel samples	AITC in the second extract, µg	Recovery of added AITC		
			AITC added, µg	AITC found, µg	% of AITC found
1	12	55·3 ± 3·6			
2	9	58·8 ± 4·9			
			18	15·7	87
			36	30·2	83

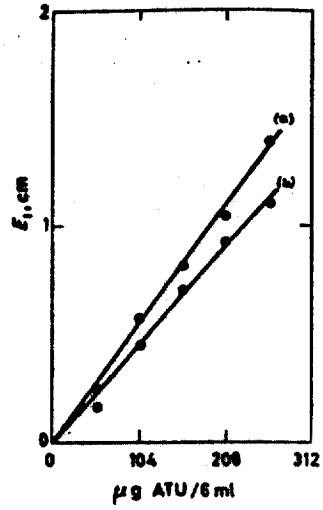


FIG. 6. *Corrected absorbance of the increasing amounts of AITC (after the conversion into thiourea)* (a) *before and* (b) *after the distillation*

By the method presented it is possible to estimate the total content of mustard oils in much smaller amounts of plant material (0·1 g of the original material) than by other methods.[4,10,19] It thus may be possible to estimate these compounds in small anatomical parts of relatively small plants. This may contribute to knowledge of the biological rôle and metabolism of these compounds in the plant organism.

Acknowledgments

The authors wish to thank Prof. A. Kjaer (Copenhagen), Dr. L. Drobnica (Bratislava) and Dr. J. Sedlák (Bratislava) for supplying some of the reference compounds.

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obtained 3 broad to 1 narrow leaf in F_2, of course, by eye estimation, and suggested a monogenic inheritance for this character, while Ramiah (loc-cit) from his studies presumed that for the leaf size in rice, the number of genes involved are not many.

Seasonal effect was found to influence the leaf size of F_2 plants and their parents alike. Late sowings always resulted in reduction in leaf length and leaf breadth. Between early and normal seasonal sowings no significant variation was recorded. From the above observations, it is suggested that for a particular variety, its optimum environmental condition is necessary for proper expression of its genes. It is interesting to note that contrary to the previous experiences, in this investigation no correlation was found to exist between width of grain and of leaf (narrower grains tend to give narrow leaved plants). Inspite of production of narrower grains the variety *Charnock* was found to bear broader leaves than that of *Patnai*-23 which produce comparatively broader grains[3].

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Detection of Hydrocyanic Acid in Edible Oils (Mustard Oil).

In place of the Prussian blue test[1] for the detection of HCN in mustard oil a new and simpler test is described.

In this note a new test based on the reaction of HCN on picric acid paper is described. No preliminary saponification of the oil is necessary.

The Test; Pour 15 to 30 *ml* of the suspected oil depending on its HCN content into a 250-*ml* conical flask and mix well with about 50*ml* water. Add 15 *ml* of 10% tartaric acid solution and mix. Stopper the flask with a velvet cork from which hangs a picric acid paper (about 3" long) previously wetted with a drop of 5% Na_2CO_3 solution. The flask is placed on a hot waterbath by the side of the steam vent and not directly on the steam, for 30-45 minutes. In presence of HCN the picric acid paper acquires red colour. Ignore pink or light reddish hue which may at times appear at the periphery of the picric acid paper. The test is very sensitive.

The picric acid paper is prepared by soaking No. 1 Whatman paper in a saturated aqueous solution of picric acid, draining the excess liquid and drying the dyed paper in air.

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The Distribution of Bound Lipids in the Neurosecretory Cells*

The stainable neurosecretory substance was considered to be a complex, consisting of the posterior lobe hormone and a glycolipoprotein by Schiebler[8,9,10]. Scharrer and Scharrer[7] endorsed the same view, but it was controverted by Sloper[11] and Howe and Pearse[6] as they could not detect the lipid moeity of the neurosecretory substance. Schiebler's view was, however, once more supported by Fraser[3]. He found that the cytoplasm of group—5 neurosecretory cells of the larva of *Lucilia caesar* was strongly and uniformly sudanophil indicating the presence of glycophospholipid or lipoprotein.

According to Berenbaum[2] "Lipids, by virtue of their hydrophobe qualities, play an important part in the make-up of natural semi-permeable membranes. In this connexion the constant association of lipid with nucleoprotein is of great interest. It is evident that the complex activities in which nucleoproteins play a vital role, such as protein synthesis or mitosis, require for their performance the orderly interaction of many cell components. It may be suggested that, for such interaction to remain orderly many efficient but labile barriers must be constantly in operation, in order to prevent interference by inappropriate cell constituents. Such barriers might be provided by lipid or lipoprotein

* Carried out by the grant in aid from Ministry of Scientific Research and Cultural Affairs, Govt. of India.

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ES

T

ed mint, but the two that consitute
re Peppermint and Spearmint.
aves and flowering tops of *Mentha*
a spicata L. Both are perennial her-
or Mint family, and both are native
spearmint are cultivated in all parts
United Kingdom, and in North and
nt sold in the United States is grown

Both have erect stems and ascending
more so than the peppermint. The
ply toothed, and dark green; those of
ightly longer and narrower than pep-
light green in color. The flowers
col hose of the peppermint are
s in the upper leaf axils, those of the
interrupted, terminal spikes. Both

oom. Generally, they are allowed to
iod of time and the drying completed
rolled, circulating warm air, avoiding
en the leaves have attained the desired
from the stems, allowed to dry out
wire sieve or fragmented mechanical-

c, sweetish, strong aroma, and an aro-
oling after taste; spearmint to a lesser

meat and fish sauces, salads, mint jelly,
ns.

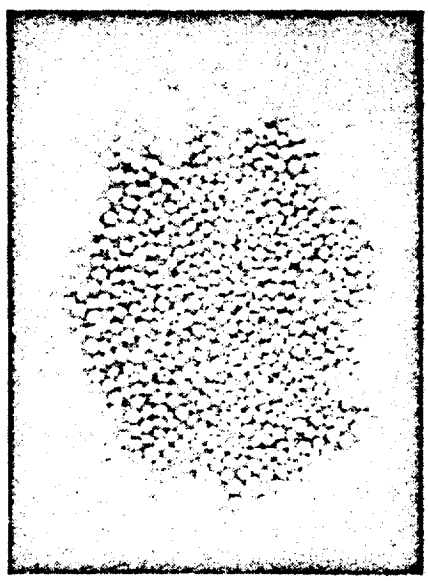

Fig. 29. Yellow (White) Mustard Seed.

MUSTARD

In spices, the term mustard refers to the seeds of two different plants of the

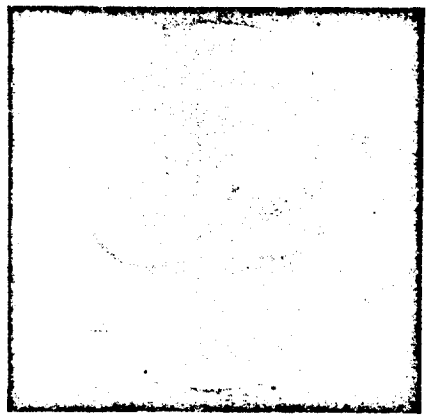

Fig. 30. Mustard Seed × 7½. *Left:* White (or Yellow). *Right:* Black (or Brown).

same family—namely, *Sinapis alba* L., from which is obtained white mustard seeds, and *Brassica nigra* (L.) Koch (also named *Sinapis nigra* L.) from which is obtained black mustard seeds. The former is commonly known as yellow mustard and the latter as brown—understandable terminology when the color of the respective seeds is considered.

Both plants belong to the *Cruciferae*, the name being derived from the cross-like figure formed by the four petals of the flower, or Mustard family.

The white mustard plant is native to southern Europe and western temperate Asia; the black mustard plant is native to central and southern Europe and western temperate Asia.

Both plants are cultivated in many parts of the world including Denmark, Holland, Germany, Austria, Italy, North Africa, India, Chile, United Kingdom, United States of America, and Canada. Most of the mustard seed imported into the U.S.A. in recent times has come from Canada.

The white mustard plant is a herbaceous annual reaching a height of about two feet. The lower leaves are cleft or parted, the upper leaves are cut somewhat into two lower lobes and a larger terminal lobe. The leaves are irregularly toothed, hairy, and somewhat tough. The flowers are small, yellow, and borne in racemes. The fruit is a cylindrical, bristly pod with a long, sword-shaped beak. The seeds are small, nearly globular, and yellowish in color.

The black mustard plant is also an herbaceous annual, reaching a height of two feet or more. The lower leaves are large, with two lower lobes and a large terminal lobe, the central leaves are moderately lobed, and the uppermost leaves are entire and narrow. The lower and central leaves are irregularly toothed. The flowers are similar to those of the white mustard plant. The fruit is a four-sided pod with a short beak. The seed is smaller than the white mustard seed, nearly globular, and dark reddish-brown in color.

The dry mustard seeds of either plant have no perceptible odor; the taste of the seeds of both plants is pungent.

Whole mustard seeds are employed in the preparation of pickles of various kinds. They are an ingredient of whole mixed pickling spice. Ground mustard is used in the preparation of mustard pickles; in the manufacture of prepared mustard—a preparation of ground mustard, white vinegar, a number of other ground spices, and salt; and in the preparation of mustard sauce, sea food sause, mayonnaise, and salad dressings. The table condiment often referred to as "hot" or "English" mustard is the product of a blend of ground white (yellow) and black (brown) mustard seeds.

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(60)

(c) Well under 100 but more than 50 colonies on 21 plates.

(d) More than 200 colonies on 20 plates.

(e) Less than 50 colonies on 13 plates.

These 100 plates were prepared from stools of individuals presenting themselves for carrier examination; none of these stools contained any mucus. The following organisms were isolated :—

Bact. flexneri—on three occasions,

Bact. typhosum—once, and

Non-mannite fermenters, indol positive—twice.

These six plates from which the above organisms were isolated all belonged to groups (a) or (b) of the above classification. Two strains of *Bact. flexneri* were isolated from (b), that is, plates on which there were more than 100 colonies but less than 200, and the rest from (a), that is, plates with approximately 100 colonies.

Control.—In a parallel series plated by the loop method no organisms were isolated.

The following are the advantages of this method :—

(1) It is standardized.

(2) If the technique is followed with even a moderate degree of accuracy, the results are good. Plating by ordinary methods requires experience.

(3) The whole plate is usefully employed.

The following are the disadvantages :—

(1) A sterile plate, or an almost sterile plate, may be obtained with annoying frequency, though this will also occur by the loop method.

(2) It takes time.

(3) It cannot of course be used for mucus-containing stools.

[*Note.*—An additional disadvantage in this method is that it demands an absolutely sterile plate, an ideal it is difficult to achieve with certainty in the hot humid plains of India. A small overlooked colony would lead to contamination of the whole plate, whereas by the loop method quite a large colony can be avoided.—EDITOR, *I. M. G.*]

A NEW CAPSULE FORCEPS

By G. JOSEPH GNANADICKAM

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WITH reference to my paper in the *Indian Medical Gazette* of October 1932, on the intra-capsular extraction of the senile cataract, I have been receiving many enquiries as to the forceps I recommend.

I was then using both Stanculeanu's and Elschnig's forceps; each had its own merits. Messrs. John Weiss and Son of London have made for me a new forceps, combining the advantages of those two forceps in one, and eliminating their respective disadvantages to some extent.

This instrument is about the size of Stanculeanu's forceps, but the blades are very like Elschnig's. The double curvature on the blades is so shaped as to give a proper grasp of the capsule in its lower portion without touching the centre or pushing the cornea too much forward. The grip is firm, but never tearing.

The blades themselves are thin, taking very little space in the anterior chamber. The size of the handles and the spring action are such that one could actually feel the force used in the pinch.

I have so far used the forceps on over 500 cases and have found it to be very satisfactory. The above illustration shows the actual size of the instrument.

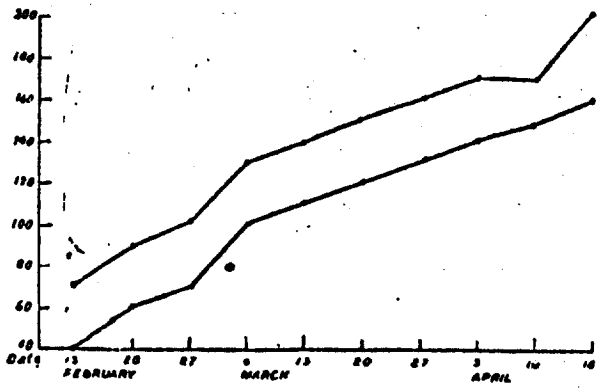
NUTRITIVE VALUE OF MUSTARD OIL

By B. B. BRAHMACHARI, D.P.H.

Director of Public Health Laboratory, Bengal

As I have shown in my article on the vitamin value of the food fats of Bengal (Brahmachari, 1932), mustard oil, *i.e.*, the oil expressed from the seeds of the mustard plants, and ghee are the only two fats which enter, as such, into the dietary of the people of Bengal, and, of the two, mustard oil is the more important. It is taken throughout the province, in towns as well as in villages, with their daily food by the rich as well as the poor, while ghee is used more in towns than in villages, and is more or less limited to the well-to-do and the middle class

Weight increase in two rats on basic diet and mustard oil *plus* spinach.



people. As a rule mustard oil is cooked with the other food articles; but occasionally it is also taken mixed with boiled potatoes and other

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vegetables, boiled eggs, roasted brinjals, etc. Though of such importance as food, it does not seem to have been studied from the point of view of its nutritive value.

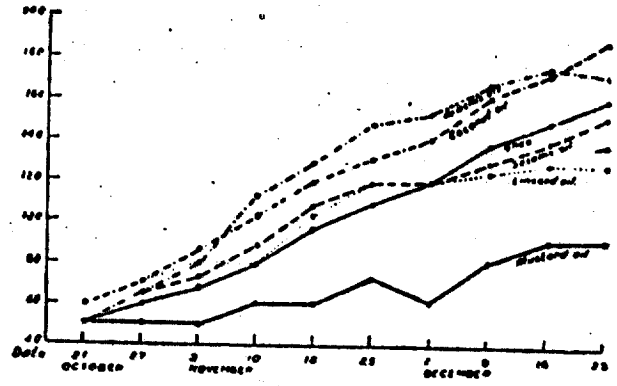
In my article referred to above, it was shown that mustard oil does not contain any vitamin A. In studying the vitamin value of the *kacha* ghee, *i.e.*, ghee that has not been hard boiled, we had to test this oil again on three rats as control. The basal diet was the same, *viz*,

Casein	19 units
Starch	55 "
Yeast	5 "
Salt mixture (McCollum and Simmonds, 1918) ..	5 "

To this was added, as the fat portion of the diet, 16 units of mustard oil.

The three rats were all of the same litter, all females, born on the 23rd May, 1932. The experiment was started on the 14th July next, *i.e.*, when they were seven weeks old and all weighed 70 grammes. They fared as follows :—

Weight increase in rats of a basic diet to which different oils have been added, as fat.



The nutritive value of mustard oil as compared with that of the other edible oils and fats.—For the experiment, we used the same diet as before, the fat being represented by the

Serial number	Weight in grammes on various dates										
	14/7	21/7	28/7	5/8	12/8	19/8	26/8	3/9	12/9	16/9	20/9
1	70	40	60	50	50	60	50	50	50	50	50
2	70	50	60	50	50	60	60	70	60	60	60
3	70	50	60	50	60	60	70	70	60	60	60

On the 20th September, *i.e.*, after a period of nine weeks, they were below the weight at which they had started and were developing xerophthalmia. As their subsequent fate was obvious, the experiment was discontinued.

respective fats under experiment. The casein, however, was not soxhleted nor heated to destroy the vitamin that might be contained in the trace of milk fat remaining in it. The result is shown in the following table :—

	Date of birth	Fat given	Weight in grammes on various dates									
			21/10	27/10	3/11	10/11	18/11	25/11	2/12	9/12	16/12	23/12
Male	18-8-32	Ghee	50	60	70	80	100	110	120	140	150	160
Female	"	"	50	60	70	80	90	100	110	110	120	130
Male	"	Arachis oil.	50	65	80	110	125	145	150	165	175	170
Female	"	"	50	65	70	80	90	100	110	120	145	littered
Male	21-8-32	Sesame oil.	50	65	75	90	110	120	120	130	140	150
Female	"	"	50	70	80	85	105	110	110	120	littered	..
Male	"	Linseed oil.	60	70	80	80	105	120	120	125	130	130
Female	"	"	40	50	60	60	70	75	90	littered	..	..
Male	"	Coconut oil.	60	70	85	100	120	130	140	160	175	185
Female	"	"	50	60	65	75	90	100	100	115	115	125
Male	"	Mustard oil.	50	50	50	60	60	70	60	80	90	90
Female	"	"	40	died 24/10	..	..	..	..	..	..	..	..

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Growth of the rats was quite satisfactory with all the fats except mustard oil. Coconut oil is shown by McCarrison (1929) to contain some vitamin A, but to be poor in it, and gingelly (*i.e.*, sesame oil), linseed oil and ground nut (*i.e.*, the arachis oil) to contain very little. So the growth under them is largely due to the trace of milk fat which was in the casein and was not removed, nor rendered inert, as in the previous experiments. If so, the poor growth under the mustard oil with the same casein is analogous to the phenomenon observed by Fridericia (1924); he found that by adding hydrogenated whale oil to a diet in which the vitamin was supplied by butter fat the vitamin A of the latter was inactivated. In this case the mustard oil appears to have inactivated the vitamin A in the casein.

The nutritive value of mustard oil when given along with sufficient quantity of vitamin A.—Spinach is known to be rich in vitamins; McCarrison shows it with three crosses (*i.e.*, rich in vitamin) under each of the three vitamins A, B and C. So I next proceeded with six rats of the same litter born on 19th December, 1932, placing them under the same diet containing mustard oil, but adding enough of spinach for vitamin A. The experiment was started on the 13th February, 1933, *i.e.*, when they were 8 weeks old, with the following results :—

	Weight in grammes on various dates									
	13/2	20/2	27/2	6/3	13/3	20/3	27/3	3/4	10/4	18/4
Male	70	70	100	120	125	125	140	150	150	170
Female	50	50	60	90	100	110	120	130	littered	..
Male	70	70	90	120	130	140	150	160	170	170
Female	50	50	60	80	85	100	110	150	littered	..
Male	70	90	100	130	140	150	160	170	170	200
Male	40	60	70	100	115	120	130	140	150	160

Conclusion.—We may fairly conclude that mustard oil—

(*i*) is entirely destitute of vitamin A,
(*ii*) tends to inactivate vitamin A in other food articles given along with it,
(*iii*) but, as fat, is as nutritive as other fats, provided sufficient vitamin A is supplied in other articles of food.

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A Mirror of Hospital Practice

A CASE OF WEIL'S DISEASE OR INFECTIVE JAUNDICE

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ASSISTANT SURGEON C. M. E. EYLES, I.M.D.
and
SUB-ASSISTANT SURGEON BACHAN SINGH, I.M.D.
Burma District, Maymyo

A BRITISH OFFICER, aged 52, stationed in Maymyo since February 1933, reported sick on the morning of the 29th September, 1933, complaining of headache and severe pain in the limbs, especially the legs.

Temperature varied between 99° and 101°F., the pulse was irregular and there were frequent extrasystoles. He fainted in the toilet room that evening, and was therefore admitted into the British Military Hospital, Maymyo, the same day.

Previous history.—He had been out snipe shooting three times at intervals of seven days, each time to the same spot, the sixth mile stone from Mandalay, east of the road. The last time was on the 27th September, 1933, *i.e.*, two days before reporting sick. He said that on that day he felt off colour and owing to pain in the legs he could not cover the usual long distances even after long intervals of rest.

Progress in hospital.—The patient ran an irregular temperature, ranging from 99° to 101°F., for four days, the pulse becoming more regular with rest. On the 2nd October, 1933, he was slightly jaundiced and the breath was offensive, and was put on fat- and protein-free fluid diet.

Urine examination: Specific gravity—1016, acid, smoky, albumin present and the deposit showed a few pus cells, red blood cells and epithelial cells.

3rd October.—Jaundice greatly increased; urine—8 ounces; sweated profusely, clothes having to be changed five times.

4th October.—Jaundice increased; bowels not opened; had not passed urine since previous midday; was put on alkaline diuretics and antiphlogistine applied over loins.

5th October.—The temperature was down to normal; he spent a restless night; bowels opened thrice after salts; stools clay-coloured; no urine passed; dry cupping to loins twice a day; catheterized urine—three ounces; blood pressure 110/73.

Laboratory findings:—

White blood cell count 16,000 per cubic millimetres
Differential count—

Polymorphonuclears	93	per cent
Lymphocytes ..	6	"
Large mononuclears	1	"

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Effect of Vegetable Oils on Tissue Lipids of Rhesus Monkeys

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Department of Physiology & Biochemistry, Sardar Patel Medical College, Bikaner

Manuscript received 8 *July* 1963

Total lipids, cholesterol and phospholipids contents of different tissues, viz. plasma, heart, thoracic aorta, abdominal aorta, liver, brain, kidney, adrenals and skin of rhesus monkeys fed sesame oil (iodine value, 110), mustard oil (iodine value, 104) and coconut oil (iodine value, 9), with or without cholesterol supplementation, for nine months have been determined. No correlation has been observed in the distribution of tissue lipids and the treatments given to the animals. While sesame oil increases cholesterol deposition in thoracic aorta and abdominal aorta, mustard oil and coconut oil diminish arterial cholesterol. The deposition of cholesterol in the arteries does not depend on the unsaturation of the oils. Cholesterol formation in brain and skin is depressed by the oils, whereas heart is affected to a less extent. Coconut oil feeding produces increased liver lipid and mustard oil fed animals have higher liver cholesterol. The effects of different oils on the tissue distribution of lipids become less clear when cholesterol is added to the diet. Tissue distribution of lipids appears to depend more on some unknown properties of the oils than on their unsaturation.

WHEN rhesus monkeys are fed vegetable oils along with cholesterol, the degree of unsaturation of the oils has been found to have no direct relationship to plasma cholesterol level[1]. On prolonged administration of the oils along with cholesterol, the rise in the plasma lipids of monkeys continued only up to the fifth month which subsequently diminished gradually. This was associated with diminished faecal excretion of sterols and bile acids[2,3]. This indicated that either the dietary fat and cholesterol are metabolized more efficiently or there could be increased deposition of these lipids into the different tissues. Gerson *et al.*[4] showed that a low fat diet supplemented with corn oil lowered the serum cholesterol but increased the cholesterol contents of heart, aorta, liver, intestine and muscles. Some recent studies also indicated the absence of any correlation of plasma cholesterol and concentration and distribution of cholesterol in other tissues[5-7]. It was, therefore, of interest to investigate the tissue distribution of total lipids, total cholesterol and phospholipids in rhesus monkeys fed different oils, with or without cholesterol supplementation, for nine months.

Materials and Methods

Rhesus monkeys (av. wt, 4 kg.) were fed a basal diet consisting of wheat flour, 64 parts; ground Bengal gram (*Cicer arietinum*), 20 parts; commercial casein, 12 parts; calcium carbonate, 3 parts; and common salt, 1 part. The diet contained fat, 2; protein, 19; carbohydrate, 65; and fibre, 1 per cent. Cholesterol level in the basal diet was 15 mg./100 g. of the diet, derived possibly from the crude casein. The animals were fed in addition 50 mg. of ascorbic acid per animal per day and 5 drops of a concentrate of vitamins A and D twice a week. The animals consumed 150 g. of the diet per day and grew normally. The animals were fed the basal diet for 3 weeks and then divided into several groups and fed different experimental diets for 9 months. The oils were fed at 10 and 20 per cent levels of the diet in order to find if increasing the quantity of oil could increase the plasma cholesterol level by increasing the intestinal absorption of cholesterol. The oils used were mustard oil (iodine value, 104), sesame oil (iodine value, 110) and coconut oil (iodine value, 9). After collecting the fasting blood samples, the animals were killed by injecting 5 ml. chloroform directly into the lungs. The death was instantaneous. The different tissues, viz. liver, brain, heart, kidney, skin, adrenal gland, thoracic aorta and abdominal aorta, were quickly removed and weighed. Skin was taken from the right hypogastric region. Adventitia were stripped off from the arteries and the intima-media preparation was used. Thoracic aorta consisted of the whole of the ascending part, arch and the upper part of the descending aorta. The tissues were cut into pieces, dried at 60°C. for 48 hr, extracted in a Soxhlet apparatus with petroleum ether for 24 hr., a portion of the ether extract evaporated and weighed to obtain total lipid, and the rest for the estimation of cholesterol[8] and phospholipid[9].

Results

The results of feeding trials indicating the effect of different diets on tissue lipid, phospholipid and cholesterol contents are given in Tables 1-3.

Effect of feeding cholesterol — Total lipids diminished in skin and adrenal glands. Phospholipids diminished in heart, kidney and adrenal gland. Cholesterol of tissues did not change appreciably.

Effect of feeding sesame oil — Total lipids diminished in skin and increased in kidney. Phospholipids increased in skin and liver and diminished in adrenals. Cholesterol increased in both thoracic and abdominal aorta and decreased in skin and brain.

Effect of feeding sesame oil and cholesterol — Total lipids diminished in skin, kidney and adrenal and increased in liver. Phospholipids increased in skin, liver and brain, and decreased in kidney and adrenal glands. Cholesterol of thoracic aorta and liver increased and that of skin, kidney and brain diminished.

Effect of feeding mustard oil — Total lipids increased in skin, kidney and adrenals. Phospholipids

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decreased in kidney and adrenals. Cholesterol increased in liver and diminished in skin, kidney, brain and adrenal.

Effect of feeding mustard oil and cholesterol — Total lipids increased in heart and liver and diminished in skin. The monkeys fed 30 g. oil showed increased phospholipids in thoracic and abdominal aorta, skin and liver and diminished phospholipids in kidney and adrenals, whereas in monkeys fed 15 g. oil phospholipids fell in the skin and increased in the brain. Cholesterol increased in the liver of all the animals, and diminished in skin, kidney, brain and adrenals. Arterial cholesterol increased in the monkey fed 30 g. oil.

Effect of feeding coconut oil — Total lipids increased in liver and kidney, and diminished in skin and adrenals. Phospholipids increased in abdominal aorta, and decreased in kidney and adrenals. Total cholesterol diminished in thoracic and abdominal aortas, skin, liver, brain, kidneys and adrenals.

Effect of feeding coconut oil and cholesterol — Total lipids increased in liver and kidney, and decreased in skin and adrenals. With the exception of one monkey fed 15 g. oil, cholesterol increased in the heart.

TABLE 1 — TISSUE LIPIDS OF MONKEYS FED DIFFERENT DIETS

(Tissue lipid values are expressed in g./100 g. wet tissue)

Diet	Heart	Skin	Liver	Kidney	Brain	Adrenal
Basal only	2·47	36·12	2·30	3·09	10·16	44·70
	5·44	41·31	3·96	3·44	9·80	32·42
Basal+cholesterol (3 g./day)	1·00	1·00	2·08	—	9·84	7·40
	4·75	7·59	3·85	2·89	9·21	22·23
Basal+sesame oil (30 g./day)	2·64	26·23	4·49	8·83	9·87	41·64
	6·67	23·36	3·57	3·62	9·58	16·90
Basal+sesame oil (30 g./day)+cholesterol (3 g./day)	3·24	9·00	4·16	2·75	10·24	21·67
Basal+sesame oil (15 g./day)+cholesterol (3 g./day)	4·07	12·61	7·21	2·76	10·23	31·60
	3·48	13·36	6·46	1·61	9·54	6·48
Basal+mustard oil (30 g./day)	2·17	3·10	2·55	2·29	9·67	16·20
	4·87	5·30	4·32	2·33	9·57	25·29
Basal+mustard oil (30 g./day)+cholesterol (3 g./day)	3·05	6·90	5·56	3·29	10·44	28·40
Basal+mustard oil (15 g./day)+cholesterol (3 g./day)	9·84	11·94	10·47	1·00	10·34	39·00
Basal+coconut oil (30 g./day)	5·62	28·05	10·71	4·88	10·59	10·45
	5·50	20·89	19·21	7·53	11·19	10·76
Basal+coconut oil (30 g./day)+cholesterol (3 g./day)	7·51	25·12	18·15	6·03	10·07	28·59
Basal+coconut oil (15 g./day)+cholesterol (3 g./day)	8·44	24·84	5·68	5·30	9·79	24·49
	2·88	24·79	14·97	7·72	10·50	—

TABLE 2 — TISSUE PHOSPHOLIPIDS OF MONKEYS FED DIFFERENT DIETS

(Tissue phospholipid values are expressed as mg. lecithin/100 g. wet tissue)

Diet	Plasma	Heart	Aorta		Skin	Liver	Kidney	Brain	Adrenal
			Th.	Ab.					
Basal only	187	567	220	184	90	1242	1395	5280	3085
	182	905	122	155	160	1212	1270	4900	5420
Basal+cholesterol (3 g./day)	181	250	70	45	38	1210	—	5150	1200
	210	332	400	325	90	1440	635	5050	1275
Basal+sesame oil (30 g./day)	158	883	78	173	245	1917	1338	4700	2425
	142	1000	145	100	240	1527	1205	5075	2378
Basal+sesame oil (30 g./day)+cholesterol (3 g./day)	280	1102	307	285	300	2050	877	6660	602
Basal+sesame oil (15 g./day)+cholesterol (3 g./day)	328	1050	110	35	168	2885	1020	7055	2020
	250	680	175	45	150	2625	670	6250	1875
Basal+mustard oil (30 g./day)	200	585	180	235	138	1111	550	4200	2250
	160	1125	160	40	150	1193	955	4840	1375
Basal+mustard oil (30 g./day)+cholesterol (3 g./day)	162	610	325	500	367	2080	968	5193	2055
Basal+mustard oil (15 g./day)+cholesterol (3 g./day)	226	865	193	195	63	1288	1200	7500	4250
Basal+coconut oil (30 g./day)	260	727	332	232	170	1090	647	4693	1525
	230	575	105	263	153	1250	675	5332	750
Basal+coconut oil (30 g./day)+cholesterol (3 g./day)	213	995	175	360	188	2005	875	5080	1650
Basal+coconut oil (15 g./day)+cholesterol (3 g./day)	339	708	87	90	350	2475	882	5325	2000
	271	1025	112	65	90	1828	975	5290	—

Th., thoracic; and Ab., abdominal.

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TABLE 3 — TISSUE CHOLESTEROL OF MONKEYS FED DIFFERENT DIETS

(Tissue cholesterol expressed in mg./100 g. wet tissue)

Diet	Plasma	Heart	Aorta		Skin	Liver	Kidney	Brain	Adrenal
			Th.	Ab.					
Basal only	138	148	154	229	335	412	492	3493	6815
	101	162	149	139	310	378	512	3639	6909
Basal+cholesterol (3 g./day)	140	105	85	166	342	362	—	3095	4259
	118	192	200	113	308	480	422	3922	8400
Basal+sesame oil (30 g./day)	136	243	173	272	180	398	332	2889	6727
	123	161	262	298	172	429	412	2282	4700
Basal+sesame oil (30 g./day)+cholesterol (3 g./day)	330	130	206	156	209	631	357	2462	6484
Basal+sesame oil (15 g./day)+cholesterol (3 g./day)	463	133	613	147	258	1453	429	2271	9950
	468	128	563	146	242	1584	385	2566	7500
Basal+mustard oil (30 g./day)	160	152	70	70	292	451	310	2322	1000
	105	144	130	134	237	549	340	2213	5916
Basal+mustard oil (30 g./day)+cholesterol (3 g./day)	210	174	475	477	279	562	360	2224	2111
Basal+mustard oil (15 g./day)+cholesterol (3 g./day)	200	124	154	182	240	513	375	2837	5461
Basal+coconut oil (30 g./day)	138	119	97	63	114	335	290	2444	772
	100	121	80	33	130	160	301	2964	370
Basal+coconut oil (30 g./day)+cholesterol (3 g./day)	245	156	471	472	220	909	364	2625	4733
Basal+coconut oil (15 g./day)+cholesterol (3 g./day)	240	120	173	173	300	759	300	2755	4111
	190	120	190	170	328	458	305	2080	—

Th., thoracic; and Ab., abdominal.

Phospholipids increased in the skin of two monkeys and in liver, and decreased in kidney and adrenal. Arterial cholesterol increased only in the monkeys fed 30 g. oil. Liver cholesterol increased in all the monkeys and diminished in kidney, brain and adrenals. Plasma cholesterol and phospholipids showed similar trends as reported previously[1-3].

Discussion

No correlation could be observed between the different groups of animals, whether fed the oils at 10 or 20 per cent levels, in the distribution of lipids in different tissues. The plasma cholesterol of monkeys fed different oils did not change appreciably whether the dose of oils was 15 or 30 g. per day. When cholesterol was added to the diet in addition to the feeding of oils, plasma cholesterol increased but the monkeys receiving sesame oil had the highest level of plasma cholesterol. Increasing the level of oil from 10 to 20 per cent in the diet did not enhance the cholesterol level further. But cholesterol-phospholipid ratio in the liver decreased in monkeys fed mustard oil and increased in animals fed sesame oil. The incorporation of fatty acids into phospholipids in the liver seemed to be more efficient in monkeys fed sesame oil than in monkeys fed mustard oil, where liver cholesterol was high. Since total cholesterol and phospholipids of liver were normal in monkeys fed coconut oil, the increased liver lipids of these animals seemed to be due to the accumulation of neutral fat. When cholesterol was added to the diet, the effects of the different vegetable oils on the tissue distribution of lipids became less clear. The distribution of lipids was not uniform or even in all the tissues. In some tissues the distribution of lipids was higher when the oils administered was 10 per cent of the diet than when the dose of the oil was 20 per cent. Though there was increase in liver lipids, the lipids content of the brain and the heart seemed to be affected to a less extent. Cholesterol formation was depressed in the brain when the diet contained high fat, with or without cholesterol. Cholesterol content of heart did not change appreciably on feeding different diets. The total lipids of heart also did not change with the exception of three monkeys fed cholesterol along with the oils. This indicated that the heart could utilize or mobilize the available fat efficiently. Popjak[10] observed mobilization of fat after cholesterol administration. The total lipid content of skin was observed to diminish in all animals after they were fed cholesterol, oils or cholesterol along with the oils. Thus, oils also had the property of increasing the mobilization of fats from the skin. Addition of cholesterol to the diet did not alter skin cholesterol level, but the addition of oils diminished it. Oils, therefore, seemed to depress cholesterol formation in the skin. Since sesame oil increased cholesterol level of both thoracic and abdominal aorta, addition of cholesterol obviously augmented the deposition. Mustard oil and coconut oil diminished arterial cholesterol. The failure to increase cholesterol of aorta after the monkeys were fed cholesterol along with the oils (15 g./day) might be due to hypocholesteremic effects of these oils on the arteries. The arterial deposition of cholesterol, therefore, did not depend on the unsaturation of the oils. Vegetable oils fed with or without cholesterol did not have any specific effect on arterial phospholipids. The changes observed in kidney and adrenal were too variable to comment. Tissue distribution of lipids seemed to depend more on the unknown properties of the oils than on their unsaturation. Plasma levels

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of cholesterol and phospholipids probably had little relation to the tissue concentration of these lipids.

Acknowledgement

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Effect of Dihydrostreptomycin on Neuromuscular Transmission in the Phrenic Nerve Diaphragm Preparation of Rat

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Rat phrenic nerve diaphragm preparation has been utilized to study the nature and factors influencing the neuromuscular blocking action of dihydrostreptomycin (DHS). In the concentrations wherein DHS exhibits neuromuscular block, the antibiotic also exerts a potent direct depressant effect on the musculature. Calcium ion concentration in the perfusion fluid influences the manifestation of neuromuscular block. Increasing the calcium concentration in the fluid diminishes the blocking effect. It is likely that only the neuromuscular blocking action is partly overcome by calcium but not the direct depressant effect on the musculature. The electrolyte concentration of other components of the perfusion fluid also appears to modify the antagonizing effect of calcium on the neuromuscular block.

ONE of the manifestations of acute streptomycin toxicity has been the respiratory depression observed both in experimental animals[1] and in post-operative clinical cases[2,3]. This depression has been attributed to the neuromuscular blocking activity of the antibiotic, since streptomycin has been shown to be capable of blocking neuromuscular conduction in experimental animals[4-6] and induce clinical disorders resembling those due to neuromuscular block[7]. But the analysis of clinical data reveal that neuromuscular block by streptomycin in the absence of other factors like administration of anaesthetics and muscle relaxants is rather rare. In an attempt to elucidate the nature of associated factors influencing the neuromuscular block, studies have been undertaken utilizing the phrenic nerve diaphragm preparation of the rat for experimentation.

Materials and Methods

Dihydrostreptomycin sulphate (DHS) was dissolved in distilled water and the required volume was added to the isolated organ bath (capacity 75 ml.), maintained at 32°C. and oxygenated continuously.

The perfusion fluid initially used (modified Ringer's) had the following composition: NaCl, 137 mmoles; $CaCl_2$, 1·3 mmoles; KCl, 2·7 mmoles; $NaHCO_3$, 12·0 mmoles; $MgCl_2$, 0·1 mmole; NaH_2PO_4, 0·42 mmole; dextrose, 2·0 g.; and distilled water, 1 litre.

In some experiments, the above solution was prepared without calcium and varying amounts of calcium were added to the bath to study the influence of calcium on the action of DHS.

Krebs' solution of the following composition was also used: NaCl, 118·0 mmoles; $CaCl_2$, 1·9 mmoles; KCl, 4·8 mmoles; $NaHCO_3$, 25·0 mmoles; $MgSO_4$, 2·4 mmoles; and KH_2PO, 1·2 mmoles; dextrose, 1·0 g.; and distilled water, 1 litre. Calcium concentration is expressed as its anhydrous salt.

The diaphragm with the phrenic nerve was prepared as per the method of Bulbring[8]. Supramaximal stimuli at the rate of 9 pulses/min. were applied.

Results

Experiments were first carried out by indirect stimulation of the muscle through the phrenic nerve in modified Ringer's fluid. Excepting a slight gradual diminution of the response, no appreciable effect was seen at 50 μg. and 100 μg./ml. of DHS during the first 3 min. Prolonged contact for 15 min. with 100 μg./ml. of DHS, during which period no stimulations were given, reduced the excitability of the muscle. The recovery after wash was quick and complete (Fig. 1). At higher concentrations a definite lessening of the height of contraction was seen. With 1 mg./ml. of DHS, recovery after wash was delayed and incomplete. At 2 mg./ml. DHS caused

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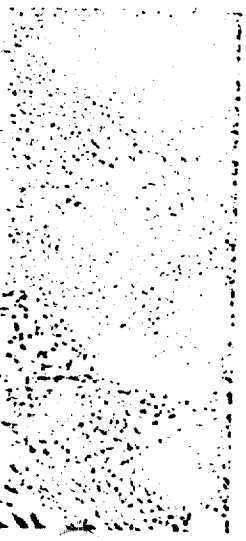

(b) s of animals
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weeks, grayish white.

Experimental Hypertension in Rats Produced by Dietary Condiments. (23029)

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Blair(1) reported that, in a group of persons undergoing routine physical examination, he had observed some degree of correlation between occurrence of hypertension with excessive use of condiments in the daily diet. He reported 50 cases, presenting the theory that excessive use of spices and condiments, especially pepper, mustard and ginger, is a common cause of hypertension; and stated his opinion that hypertension may be produced experimentally in animals by repeated feeding of these spices and condiments, and also that the damage produced may be irreversible. In pilot experiments (in cooperation with J. E. Owens, unpublished data) it was found that rats, maintained on diets to which pepper, mustard and ginger had been added, developed hypertension. However, due to circumstances beyond his control, this work was not pursued further. Personal correspondence led to resumption of our studies on experimental hypertension.

Methods. Female rats (200-250 g) were used. Blood pressure was determined by photoelectric plethysmographic method of Kersten *et al.*(2). Each value recorded represents the average of at least 4 consistent readings in a single test period. Initially, the condiments were mixed in powdered breeder's diet. Later, for reasons to be discussed, a

TABLE I. Systolic Blood Pressure of Rats on Diets Containing Condiments.

Diet	Mean systolic blood pressure* (mm Hg) ± S.E.			
	Initial	7 mo	10 mo	12 mo
Control	122 ± 7.5		115 ± 7.1	
I		154 ± 11.9	122 ± 11.4‡	174 ± 7.6†
II		152 ± 9.7	165 ± 6.3	156 ± 10.4
III		171 ± 7.4	173 ± 15.0	164 ± 4.5

* Blood pressure averages from 5-7 animals, except (†). Value for each animal an avg of at least 4 consistent readings.
† Avg of 2 animals, only survivors at 12 mo.
‡ Only experimental value not significantly different at 1% level.

commercial producer* cooperated in supplying a standard diet in which the condiments were mixed before extrusion of the pellets. All condiments were standard table grade, obtained from a wholesale grocery supply house. Diet I contained 2% pepper, Diet II contained 1% each of mustard and ginger, and Diet III contained 2% pepper, 1% mustard and 1% ginger. Blood pressures and weight of each animal were obtained periodically. At the end of one year, representative animals were sacrificed for a complete gross and microscopic histopathological study.

Results. The original powdered diets produced a high incidence of respiratory involvement, presumably because of irritant qualities of the powdered condiments, which were inhaled during feeding. Before the pellets were obtained, several deaths with pulmonary symptoms occurred. This respiratory involvement was no longer observed when the pellet diet was instituted.

Table I shows average blood pressure reading obtained during the experimental period, for all diets. The average control value obtained before diets were instituted was 122 mm Hg ± 7.48. One group was maintained continuously on Diet I (pepper) for one year. It will be noted that there was a decline in blood pressure level at 10 months to essentially normal values. From then until the end of the year all rats lost weight, 3 died, and the 2 which survived were sacrificed for pathological study. Their final pressures before autopsy were 168 and 180 mm Hg.

Another group was maintained on Diet II (mustard and ginger) for one year. These animals showed a similar rise in blood pressure (Table I), but did not exhibit a secondary decline as observed with Diet I. They did not lose weight, and appeared healthy through the experimental period.

Animals on Diet III (pepper, mustard and ginger) were started earlier, and were studied in somewhat greater detail. The average blood pressure after only 8 weeks on the diet was 167 mm Hg. Withdrawal to non-condiment control diet for 3 weeks was accompanied by a decline to 157 mm Hg. Return to Diet III for 5 weeks elevated the pressure to 168 mm Hg. Thereafter, temporary withdrawal did not reverse the pressure trend and after a cumulative total of 8 more weeks of intermittent use of Diet III the average blood pressure was 176 mm Hg. At that point the group was permanently withdrawn and kept on control diet. Throughout a period of 7 months the mean blood pressure values averaged 171 mm Hg (range 165-18[illegible]) over 14 periodic readings. The last reading at 16 months was 172 mm Hg. This implies that a permanent hypertensive state had been established. The use of experimental antihypertensive drugs produced a lowering of blood pressure to the same extent as obtained in rats with experimental renal (Grollman) hypertension.

Pathologic findings. Kidney samples from each diet series were submitted for histological examination. Two rats were from Diet I, 3 from Diet II and 3 from Diet III.

Grossly, kidneys from rats fed the above mentioned diets exhibited some congestion but were otherwise essentially negative. There was no evidence of cortical atrophy, surface scarring or vascular sclerotic changes. Blocks of tissue from these organs were fixed

* The Vi-D-Co., Marion, Ind.

in formalin and stained with hematoxylin and eosin for general topography, with Schiff's Periodic Acid for changes in basement membrane and capsule, and with Unna's Orcein stain for changes in elastica of blood vessels.

Microscopically, the parenchyma, stroma and the renal-vascular apparatus exhibited no changes compatible with any drug action. The tubular system was essentially negative, no abnormal changes were seen in the elastica of blood vessels and the PAS reaction was within normal limits. Final diagnosis was "essentially negative kidney findings."

Discussion. It would appear that mustard or ginger contains some factors capable of producing changes leading to a hypertensive state in the rat, while pepper has a factor which is less effective, though possibly additive when combined with other condiments. As a collateral observation, it was noted that the fur of the animals on Diets II and III was much softer to the touch than normal rats, while those receiving pepper failed to show this effect. The benefit probably derived from additional sulfur available in the mustard. All groups exhibited a temporary reluctance to accept the diets, but soon resumed normal intake as judged by an essentially normal growth curve. The latter part of the year for the pepper group was the only exception to this, and the cause of their decline is not known at present.

Future experiments may explore the possibility of extraction of the condiments in an attempt to isolate a single identifiable factor which will induce the hypertensive state.

In view of the essentially negative findings in the examinations of the kidneys, the authors are still at a loss to explain the mechanism of action in the production of hypertension.

Summary. Continued feeding of pepper, mustard and ginger in various diet combinations results in appearance of a statistically significant hypertension in rats. The greatest elevation occurred in a diet containing all 3 condiments; the hypertension from this diet persisted for months after the diet was discontinued. Other combinations were less effective, and work is continuing to determine if any single representative contains the necessary and sufficient factor for the production of hypertension.

The authors gratefully acknowledge the cooperation of R. J. Stein for his pathologic evaluation of the kidneys, and M. R. Sisler for technical assistance.

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From the Pharmacological Institute of the University of Freiburg i.Br.

Effect of Iodine Tincture and Mustard Oil on the Gas Exchange and Blood Circulation through the Skin

By Dr. Ernst Sametinger

(Includes 7 figures) - (Received on Oct. 27, 1930)

While numerous observations are at hand on the anatomical changes which occur in response to a local inflammatory stimulus (1), the chemical processes in the irritated tissue are less known. Their quality as well as quantity are of speci interest, however, since it was found that another pathological tissue, the carcinoma tissue, differs qualitatively and quantitatively from normal tissue in its metabolism. To this must be added the practical consideration that on the one hand the creation of local stimuli and inflammations serves a variety of therapeutic purposes, but that on the other hand the irritated and inflamed tissue itself is the object of therapy, and yet the chemical aspect of this modified stat is not sufficiently known.

The present tests are intended as a contribution to the clarification of this problem. The chemical processes were measured on the gas exchange (oxygen consumption and release of carbon dioxide). On the living animal this was possible only with simultaneous measurement of the circulation of blood through the organ to be examined. This was the dog's ear. The irritants were iodine tincture and mustard oil, and for comparison capsicum tincture, turpentine oil, and xylol wer used.

Gas exchange after local stimulation has been examined before by Gessler (2), by Groll and co-workers (3) and by Bricker and co-workers (4). Gessler produced a local necrosis by (subcutaneous) injection of 0.2-0.3 cc of mustard oil or for ic acid under the abdominal skin of a pig, and after 18-24 hours he excised from the hyperemic corona of the necrotized area a piece of skin with adhering subcutaneous connective tissue. The oxygen consumption of this excised skin fragment

measured by the method of Barcroft and Warburg, was increased as much as 70%, while that of the necrotized tissue was decreased. With the same test arrangement Groll (3) examined the oxygen consumption of severed mouse ears after exposure to various substances causing inflammation. After application of croton oil, the oxygen consumption of the mouse ear was increased during the first hour then dropping to 0 in the course of a gradually occurring necrosis within 24 hours. After application of low concentrations of diphenyl arsenic chloride, th oxygen consumption was increased, after higher concentrations, reduced. A cantharide extract low in alcohol only slightly increased the oxidation of the isolated mouse ear. Bricker (4) compared on the rabbit the ability of venous blood to bind carbon dioxide in a sound and an inflamed ear. After a burn or after exposure to croton oil, the ability to bind carbon dioxide remained unchanged. In other experiments Bricker compared the content of oxygen and carbon dioxide in the venous blood of a normal and of an inflamed rabbit ear and tried to infer therefrom the amount of gas exchange in the inflamed tissue. But these results are not unambiguous.

Method

Preliminary tests were made on rabbits, but they are not suitable because it was not possible to maintain these animals at a constant body temperature under narcosis during the test period of 6 hours. Also, rabbit ears serve for heat regulation and therefore show a greatly varying blood circulation. Moreover, the total quantity of blood is too small in them for continuous removal of blood for the purpose of the gas analysis. The tests were continued, therefore, on dogs weighing 15-40 kg, also for the reason that the blood circulation through the ear vein can be measured with the thermoflowmeter only on large animals.

The measuring of the total blood circulation through the ear required in the first place an orientation about the location of the ear veins of the dog (fig. 1 For this purpose first the point where the main ear vein enters into the vena maxillaris interna was located. A section extending caudally from the angle of the jaw parallel with the longitudinal axis of the neck exposed the vena maxilla

is interna. Coming from the trough between the easily palpable transverse process of the atlas and the auricular process, the vena auricularis posterior ente into the vena maxillaris interna from behind. The latter was tied cranially of t entrance of the ear vein, as were all its side branches to the vena maxillaris externa. Now only venous blood of the ear was present up to the confluence of the vena maxillaris externa and the vena maxillaris interna to the vena jugularis externa. Then the vena maxillaris externa was prepared free over a length of 3-5 cm, its plexus was tied here and its side branches were excluded. For the removal of blood, as wide as possible a quartz cannula was inserted through the stump of the vena maxillaris external up into the vena jugularis externa, usually making it necessary to overcome a venous valve (fig. 1).

Fig. 1. Anatomy of the veins of the dog ear.

To pipette or syringe - Cannula in V. max. ext.

The circulation of blood through the ear was measured either surgically accord ing to Barcroft and Brodie (5) or non-surgically with the thermoflowmeter of Rein (6). The first method was to tie the vena jugularis externa for the duration of one blood removal shortly after the confluence of vena maxillaris externa and interna, so that now the entire ear vein blood took its course from the vena maxillaris interna backward through the quarts cannula into a 10-cc graduated pipette connected by a rubber tube. A blood volume of 5-10 cc was clocked with the stopwatch. If the inflow of the blood was irregular, the measurement of the flow was repeated.

In non-surgical measurements with the thermoflowmeter, the diathermy thermoelement of a diameter of 1.2-2.0 mm was applied to the vena maxillaris interna in all tests. The thermo-electric recording of the blood circulation was done with a Zeiss loop galvanometer. The thermoflowmeter was calibrated surgically after each test on the same animal with the same position of the element and with the same heating intensity as in the test itself.

The gas exchange of the ear results from the amountof blood circulation and

the difference in the content of oxygen and carbon dioxide in the arterial and venous blood. When the blood circulation was determined according to Barcroft a Brodie, the blood which had flown from the quartz cannula into the graduated pipette was used for the gas analysis. When the blood circulation was determined with the thermoflowmeter, a portion of the blood arriving via the vena maxillaris interna from the ear was removed by suction through the lateral vena maxillaris externa with a paraffined, air-free syringe of 10 cc. This latter method avoids the temporary tying of the vena jugularis externa and thus better preserves the natural circulation conditions. It permits removing only a portion of the blood flowing past, so that measurement of the organ gas exchange over a prolonged period (about 1 minute) is possible. The arterial blood was taken from the arteria saphena.

Portions of 5.0 cc each of the arterial and venous blood were transferred into small vessels which had been filled with 0.5 cc of a 4% sodium fluoride solution and with mercury. The details of the filling and preservation by this method have been described by Janssen (7). The blood was kept in the vessels until the time of the blood gas analysis for at most 2 hours refrigerated at -3.0°C. Then each 1.0-cc sample was tested by the combined method of van Slyke for oxygen and carbon dioxide.

The difficulty of exact determination of the organ gas exchange from the blood circulation and from the gas content of the blood resides in that sometimes the circulation in the organ is altered still during the taking of the blood, an error which must reappear in the calculated values of the organ gas exchange. This error was examined separately, in that both the inflow of blood into the graduated pipette and the intake of blood into the syringe, as well as the undistrubed flow existing before and after blood removal were checked by continuous recording with the thermoflowmeter. It was found that the normal conditions of blood circulation through the ear are least disturbed when the graduated pipette is placed at the level of the vena jugularis externa. The deviations resulting

from letting the blood flow into the graduated pipette are entered in Table 1. They were at most +25 and -42.3% (average 18%) of the blood circulation prevaili in the ear before and after the removal of blood. The deviations of blood remov with a syringe and measurement of the blood circulation with the thermoflowmeter were at most +61 and -24.3% (average 8.6%, see Table 1, p. 5).

The error which results for the calculation of the gas exchange of the ear from the blood circulation during removal of blood has been taken into account in the following graphs, because I calculated the gas exchange of the ear from both values, normal and disturbed blood circulation,and from the gas content of the arterial and venous blood, and have shown them in the form of triangles on th columns of oxygen consumption and carbon dioxide release. The error width thus calculated is small in comparison with the variations of the gas exchange by iodine tincture and mustard oil.

The long duration of the tests averaging 10 hours required a careful treatment of the animals. Among various drugs, numal proved best as narcotic because of its long, even effect up to 24 hours, as it barely changes the body temperatu and blood pressure. Each dog received an intravenous injection of 0.5-0.75 cc per kilogram. During the test, the animals were covered up to the lower edge of the neck with a box, which was maintained at a constant temperature of 28°C by carbon filament lamps. They were protected from the radiant heat by a wool blanket.

Table 1.

Variations in rate of flow by sampling with the syringe and with the pipette. Error calculated in per cent of the undisturbed flow prevailing before sampling, where + indicates acceleration, - retardation.

Blood circulation before sampling cc/min	Blood circulation during sampling With syringe cc/min	Error ± %	With pipette cc/min	Error ± %
30	30	0	37.5	+25
30	29	-3.3	37.5	+25
26	23	-11.5	15.0	-42.3
53	53	0	52	-2
53	54	+2	32	-39.6
29	29	0	19	-37.6

Table 1 continued

Blood circulation before sampling cc/min	Blood circulation during sampling With syringe cc/min	Error ± %	With pipette cc/min	Error ± %
57	50	-12.3	57.5	+ 1.2
57	57	0	57	0
44	40	- 9	40	- 9
32	30	- 6.2	30	- 6.2
50	50	0	56.6	+13.2
43	43	0	43	0
37	28	-24.3	32	-13.5
36	30	-16.6	32	-11
16.5	17	+ 3	14	-15.1
18	29	+61	18	0
22	22	0	14	-36.3
14	15	+ 7.1	15	+ 7.1

After sampling of the arterial blood, the rectal temperature and blood pressu were taken (with a Hg manometer on the arteria saphena).

Results

1. Continuous recording of blood circulation without gas analysis

The blood circulation curves obtained by continuous recording with the thermo flowmeter for 6 hours give an almost consistent picture (see fig. 2).

In eight normal tests the blood circulation is 20-30 cc per minute. It fluctuates within moderate limits and decreases a little in the course of 6 hours. This behavior is probably attributable to changes in the systemic circulation, because blood pressure and rectal temperature increase a little as the narcosis tapers off, accompanied by a vasoconstriction which takes place also in the skin

In comparison with the normal blood circulation, after brushing the outside of the dog's ear with various local irritants there appears at first a decrease of the blood circulation lasting only minutes, followed approximately for 1 hour by an acceleration of the blood flow, later by a slowing thereof prolonged for hours. The initial decrease in blood circulation after application of the irritant is caused by reflex. It must be regarded, in the case of mustard oil, as a pain reflex, for it is accompanied by increased blood pressure and deeper respir ation. With the other agents, the chilling due to evaporation may constitute a cause of irritation. The then following acceleration of the blood circulation i not to be considered as a reflex and depends, as later tests will show, on the

chemical changes in the tissue. In part, a certain parallelism with the carbon dioxide release of the irritated tissue was detectable. In some of the tests, this normally always clear reaction of the vessels does not occur, although the reflex excitation was clearly developed, namely in the tests where the normal blood circulation through the ear was already very high and probably could not be increased further. Subsequently the blood circulation sinks to or below the normal value, although the chemical reactions of the irritated tissue have not regained their normal state.

Fig. 2. Blood circulation through dog ear in cc per minute. (Broken lines) = normal (3 tests). (Solid line) = after brushing with mustard oil (S), (5 tests).

Fig. 3. Blood circulation through dog ear in cc per minute. --- = normal. ____ = after brushing with iodine tincture (J), (3 tests).

Fig. 4. Blood circulation through dog ear after brushing with xylol (X), turpentine (T) and capsicum tincture (C), (each 1 test).

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2. Analysis of the gas exchange of the ear before and after inflammation

In an interval of 6 hours the normal ear of the dog does not have a perfectly uniform gas exchange (see fig. 5). The oxygen absorption during this time is quite constant and shows at most an increase by 50%. The values of carbon dioxide release, instead, vary more. Taking the ratio of carbon dioxide absorption to oxygen release in the sense of the respiratory quotient, in the normal state $\frac{CO_2}{O_2} = 1$ is found only as an exception. Usually the values of R.Q. are greater or less than 1.0, preferentially greater than 1.0. Rarely was R.Q. found to be 2.0, once 0.5. These fluctuations are not attributable to an incomplete blood circulation or to blockage, as they appear also in tests where the blood circulation was checked continuously with the thermoflowmeter and was found unchanged.

The shifts in the gas exchange after mustard oil and also after iodine tincture are greater than the normal fluctuations. Observation of the blood circulation through the ear under the influence of the local irritants had made it [illegible] apart from the short pain reflex: one with

increased blood circulation during the first hour, and a second after the first hour, when the increased blood circulation gradually decreases again. The blood samples which were to give information on the amount of the gas exchange were, f that reason, distributed over these two stages. In each test the normal gas exchange was determined first.

Fig. 5. Normal gas exchange of the dog ear in the course of 6 hours. (Solid) = Oxygen absorption. (Blank) = Carbon dioxide release. Bldr. = Blood pressure. Dbl. = Blood circulation. Rt. = Rectal temperature. (2 tests).

The application of mustard oil (see fig. 6) brings about in three tests immed iately after the brushing on a decrease of the oxygen consumption to 1/2 - 1/3. Only in one experiment the oxygen consumption remains unchanged. The release of carbon dioxide is always increased at the same time 2-5 times, both in compariso with the oxygen absorption and with normal carbon dioxide release. In the second stage, after 3-4 hours, one finds instead a greatly increased absorption of oxygen and a much reduced carbon dioxide release, which often drops below the normal values. Mustard oil, therefore, causes great alterations of the gas exchange in the dog ear, the oxygen absorption and carbon dioxide release moving i opposite directions.

Iodine tincture, however, affects the gas exchange differently. The oxygen consumption is increased during the first 6 hours, and if a case arises where th carbon dioxide release is slightly increased,the oxygen absorption increases sim ultaneously. The release of carbon dioxide approximately matches the amount of oxygen consumption.

Fig. 6. Gas exchange of the dog ear after brushing with mustard oil (arrow). Legend as in fig. 5. (2 tests)

Fig. 7. Gas exchange of the dog ear after brushing with iodine tincture (exp A). In experiment B first iodine tincture (1 arrow) and then mustard oil (2 arrows) was brushed on. Legend as in fig. 5 and 6.

As it may be concluded from the response of the oxygen consumption and of the carbon dioxide release that after the application of mustard oil or iodine tincture qualitatively as well as quantitatively different metabolic processes occur it was of interest to see how the gas exchange of the tissues would respond to a combination of the two actions. I therefore produced iodine tincture effect on dog ear and then also applied mustard oil. The effect typical of iodine tincture namely an increase of the oxidations to double the normal value, is suppressed by the subsequent mustard oil effect. The carbon dioxide production goes up in the manner seen to be typical of mustard oil.

If the effects of the two substances, iodine tincture and mustard oil, were qualitatively alike, an addition of the effects should have occurred. But since it is possible to alter the gas exchange after iodine tincture completely with mustard oil, this proves also a different effect of the two substances on the tissue cells.

Concerning the relationshp between the gas exchange of the ear, the blood circulation through it, and the externally visible changes, I have observed the following: The first blood sample was taken 7-30 minutes after the irritant had been brushed on. At that time only a reddening of the ear could be seen externally, without roughly measurable increase in thickness. Mustard oil had caused an acceleration, but iodine tincture no substantial changes in the blood circulation. During the subsequent second removal of blood, the externally visible hyperemia had usually completely vanished, the rate of flow of the blood had dropped to or below the normal value. The ear brushed with mustard oil had assumed double its original thickness, a tightly filled edema had formed. After iodine tincture application, no increase in thickness was observable outwardly after 3-6 hours. During this time the ears were examined histologically several times. As has been described also by Fritzler (8) on guinea pig skin, they showed all signs of an inflammation: After iodine tincture light edema and minor cell proliferation, after mustard oil severe edematous lesions and ample cell proliferation. I did not examine the ears microscopically at the time of the first blood removal, as Fritzler observed the first morphological changes

only after 1 hour.

I summarize my observations as follows. Iodine tincture increases the oxygen absorption alone or the oxygen absorption and the carbon dioxide release simultaneously and in the same degree during an observation period of 6 hours. Observed outwardly, the skin is reddened more, but the quantity of blood flowing through the ear is insignificantly accelerated. The ŒHematization is slight during the 6-hour observation period.

Mustard oil reduces the oxygen absorption by 30-50% while simultaneously the carbon dioxide release is greatly increased within the first hour. During this time the blood circulation is increased, the skin is reddened, but an edema is not yet observable. In the course of the next 2-5 hours, the oxygen absorption increases beyond the normal values, while at the same time the carbon dioxide release falls off sharply. In this state a severe edema exists and the blood circulation has dropped to or below the normal value. In my experiments the action of both substances was reversible; it abated after a few days. Compared with the findings obtained by Gessler, Groll and myself, there was agreement in the fact that some hours after the inflammatory irritation the oxygen requirement in the tissue is increased. Since in this stage cell proliferation already exists, it follows that in tissue irritated by inflammation the cell proliferation is accompanied by increased oxygen consumption and therein differs qualitatively fr the tumor tissue which, according to Warburg, lives predominantly at the expense of enzymatic processes without oxygen consumption. Continuous testing of oxygen absorption and carbon dioxide release beings into evidence the increase of the oxygen requirement as a result of preceding chemical processes in which the oxygen absorption is restricted and the carbon dioxide release considerably increased. Whether organic acids as products of damaged cells with damaged oxidation are of importance here could not be seen from my experiments and requires further investigation.

The processes of the tissue gas exchange just described apply only to the action of mustard oil. The effect of iodine tincture is different and consists in

a gradual uniform increase in the oxygen absorption and carbon dioxide release. Whether this different behavior is qualitatively or only quantitatively different only a finer analysis of the metabolic processes can decide.

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