SORBIC ACID

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Sorbic Acid-Hard Copy-Volume 1 #4619

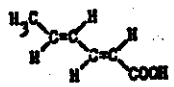
No Date

SORBIC ACID

Chemical names Sorbic acid; Trans, trans-2,4-hexadienoic acid

Empirical formula $C_6H_8O_2$

Structural formula



Molecular weight 112.13

Definition Sorbic acid, after drying for 4 hours in a vacuum desiccator over sulfuric acid, contains not less than 99 per cent. of $C_6H_8O_2$.

Description Sorbic acid is a white, crystalline solid with a mildly acrid odour.

Uses As an antimicrobial and fungistatic agent.

Biological Data

This additive was evaluated by the Joint FAO/WHO Expert Committee on Food Additives in its Sixth and Eighth Reports (FAO/WHO, 1962; FAO/WHO, 1965). Since their publication some new experimental work has been carried out on this compound. This and other work not included in the previous reports is presented and discussed in this monograph.

Biochemical aspects

The metabolism of 1-^{14}C-sorbic acid has been studied by Fingerhut et al. (1962); 85 per cent. of the activity was found in the expired CO_2, 0.4 per cent. in the faeces, 2 per cent. in the urine as urea and CO_2, 3 per cent. in internal organs, 3 per cent. in the skeletal muscles and 6.6 per cent. in the other parts of the carcass. No glycogen was formed from sorbic acid. Most of the activity was found in the subcutaneous fat deposits and in the lipids of the organs. There was a linear relationship between dose and oxidation rate; the half-life of the oxidation was 40-110 minutes in the dose range from 60-1200 mg/kg body-weight. In a similar experiment on mice, also using 1-^{14}C-sorbic acid, these results were confirmed; 81 ± 10 per cent. of the sorbic acid was oxidized to CO_2, the dose given ranging from 40-3000 mg/kg body-weight. About 7 per cent. of the activity was excreted as sorbic

[illegible] per cent. as trans, trans-muconic acid (Westöö, 1964).

[illegible] studies

The feeding experiment on groups of 100 rats (50 males and 50 females) given 0 and 5 per cent. sorbic acid was extended to the whole life-span of the first generation. The average life-span of the group receiving sorbic acid was 811 days for the males and 789 days for the females. In the control group the life-span of the males was 709 days and for the females 804 days, possibly suggesting protection by sorbic acid against lung infection. Autopsies were performed on all rats of the first generation that died during the experiment. There were no differences in the organ weight of the individual groups nor in the distribution of the causes of death. In each group (5 per cent. sorbic acid and controls) only 2 tumours were found. The animals of the second generation were sacrificed after 250 days of feeding sorbic acid. Examination of liver, kidney, heart and testes showed no abnormalities (Lang, 1962; Lang et al., 1967).

Evaluation

(See Potassium Sorbate)

REFERENCES

FAO/WHO (1962) FAO Nutrition Meetings Report Series No. 31; Wld Hlth Org. techn. Rep. Ser., 228

FAO/WHO (1965) FAO Nutrition Meetings Report Series No. 38; Wld Hlth Org. techn. Rep. Ser., 309

Fingerhut, M., Schmidt, B. & Lang, K. (1962) Biochem. Z., 335, 118

Lang, K. (1962) Arzneimittelforsch., 10, 937

Lang, K. et al. (1967) In preparation

Westöö, E. (1964) Acta chem. scand., 18, 1373

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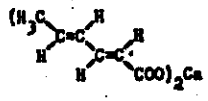
CALCIUM SORBATE

Chemical names Calcium sorbate, Calcium salt of trans,trans-2,4-hexadienoic acid

Empirical formula $C_{12}H_{14}CaO_4$

Structural formula



Molecular weight 262.32

Definition Calcium sorbate contains not less than 98 per cent. and not more than the equivalent of 102 per cent. of $C_{12}H_{14}CaO_4$ after drying.

Description Calcium sorbate occurs as a fine white crystalline powder.

Uses As an antimicrobial and fungistatic agent.

Biological Data

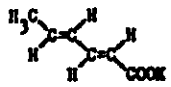
(See Potassium Sorbate)

POTASSIUM SORBATE

Chemical names Potassium sorbate; Potassium salt of trans,trans-2,4-hexadienoic acid

Empirical formula $C_6H_7O_2K$

Structural formula



Molecular weight 150.22

Definition Potassium sorbate contains not less than 98 per cent. and not more than the equivalent of 102 per cent. of $C_6H_7O_2K$.

Description Potassium sorbate occurs as white or yellowish-white crystals or crystalline powder.

Uses As an antimicrobial and fungistatic agent.

Biological Data

Biochemical aspects

The studies on the metabolism of ^{14}C-labelled sorbic acid were performed by using either sorbic acid neutralized with sodium hydroxide (Fingerhut et al., 1962) or the potassium salt (Westöö, 1964).

Acute toxicity

Substance	Animal	Route	LD_{50} (mg/kg body-weight)	References
Sodium sorbate	rat	oral	7 160	Smyth & Carpenter, 1948
Sodium sorbate	rat	oral	4 000 and 5 940	Deuel et al., 1954
Potassium sorbate (solid isomer)	rat	oral	4 920	Mellon Institute, 1954
Potassium sorbate (mixed isomer)	rat	oral	6 170	Mellon Institute, 1954
Sodium sorbate	mouse	i.p.	2 500	Rhône-Poulenc, 1965
Potassium sorbate	mouse	i.p.	1 300	Rhône-Poulenc, 1965

Short-term studies

Rat. Groups of 10 rats (5 male and 5 female) were fed potassium sorbate (solid or mixed isomers) at levels of 0, 1, 2, 5 and 10 per cent. of the diet for 3 months. Body-weight gain was initially depressed at 10 per cent., and, to a lesser degree, in the 5 per cent. female group. There are no figures given for the food consumption during this time. At the end of the experiment, the weights of the rats receiving 10 per cent. of either isomer were slightly depressed, but the food consumption of these animals was smaller. The food efficiency (weight gain/g food) was practically the same as in the control group. Relative liver weights were the same in all groups. Kidney weights were increased at the 10 per cent. level, probably due to the high potassium load. This was also noted to a lesser degree at the 5 per cent. level. Gross pathological examination showed no abnormalities, even in the 10 per cent. level [illegible] (Mellon Institute, 1954).

Dog. Eight dogs received 1 per cent. and 8 dogs 2 per cent. of the salt or mixed isomers of potassium sorbate in the diet for 3 months; 4 dogs were used as controls. There were no differences in weight gain. Gross examination on autopsy showed no evidence of a deleterious effect attributable to the sorbates (Mellon Institute, 1954).

Comments

The level of 10 per cent. and even 5 per cent. potassium sorbate causes a very high potassium intake. The food consumption of 15 g/rat/day results in a daily intake of 12 meq. K for the 10 per cent. level group. The normal daily allowance for the mature rat is 1.5 meq. K.

When considering the results of the short-term studies, allowance has been made for the adverse effects of high potassium intake. Accordingly, it was concluded that the same no-effect level could be accepted for evaluation of sorbic acid and its Ca and K salts.

It is desirable that long-term studies be carried out on animals and metabolic investigations in man using the Ca or K salts.

Evaluation

Level causing no toxicological effect

Rat. 50 000 ppm in the diet, equivalent to 2500 mg/kg body-weight/day.

Estimate of acceptable daily intakes for man

	mg/kg body-weight[1]
Unconditional acceptance	0-12.5
Conditional acceptance	12.5-25

REFERENCES

Deuel, H. J. jr et al. (1954) Food Res., 19, 1

Fingerhut, M., Schmidt, B. & Lang, K. (1962) Biochem. Z., 366, 118

Mellon Institute (1954) Unpublished report

Rhône-Poulenc (1965) Unpublished report

Smyth, H. F. jr & Carpenter, C. P. (1948) J. Industr. Hyg. Toxicol., 30, 63

Westöö, G. (1964) Acta chem. scand., 18, 1373

[1] As the sum of sorbic acid and Ca and K sorbates (calculated as sorbic acid).

SULFUR DIOXIDE AND RELATED SUBSTANCES

SULFUR DIOXIDE

Chemical name: Sulfur dioxide; Sulfurous acid anhydride

Chemical formula: SO_2

Molecular weight: 64.07

Definition: Sulfur dioxide contains not less than 95 per cent. SO_2.

Description: Sulfur dioxide is a colourless, nonflammable gas, with a strong, pungent, suffocating odour.

Biological Data

(See Sodium Sulfite)

POTASSIUM METABISULFITE

Chemical names: Potassium metabisulfite; Potassium pyrosulfite; Potassium disulfite

Empirical formula: $K_2S_2O_5$

Molecular weight: 222.33

Definition: Potassium metabisulfite contains not less than 90 per cent. of $K_2S_2O_5$.

Description: Potassium metabisulfite occurs as colourless free-flowing crystals, crystalline powder, or granules, usually having an odour of sulfur dioxide.

Biological Data

(See Sodium Sulfite)

SODIUM HYDROGEN SULFITE

Synonyms: Sodium bisulfite; Sodium acid sulfite

Chemical name: Sodium hydrogen sulfite

Chemical formula: $NaHSO_3$

Sorbic acid

R. E. J. Lishmund, chemicals division, Hoechst UK Ltd, discusses the non-toxicity and acceptability of sorbic acid in the framework of the current UK food regulations.

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Formerly the use of preservatives in the UK was governed by *The Public Health (Preservatives, etc., in Food) Regulations*, enacted in 1925. Amendments followed in 1926, 1927, 1940, 1948, 1953 and two in 1958.

In 1947, a Food Standards Committee was set up which in turn appointed a Preservatives Sub-Committee in January 1951, whose task it was to review the then current regulations and make recommendations for their amendment. In 1954 this Sub-Committee reported on anti-oxidants, colouring matters in 1954 and 1955, emulsifying and stabilising agents in 1956 and finally on preservatives in November 1959. Three years later these recommendations became embodied in *The Preservatives in Food Regulations*, 1962.

Over [illegible] years passed between the setting up of the Preservatives Sub-Committee and the publishing of the regulations in July 1962 although some amendments to the 1955 regulations were made in the meantime. Even three and a half years elapsed between the Preservatives Sub-Committee Report and the passing of the 1962 regulations. The Preservatives Sub-Committee recommended "that some suitable machinery be created for keeping the situation under review and for advising on such changes in the law as may be necessary from time to time". In spite of this, already seven years have gone by and the Food Additives and Contaminants Committee (FACC) of the Ministry of Agriculture, Fisheries and Food is only now still deliberating on advice to the Minister concerning amendments to the 1962 regulations.

Will history repeat itself with many years passing before a new Report proposing amendments is published, and even then those amendments only coming into force three or four years later still? If so, the UK food industry will have been denied the advantage of technological advances in food preservation for over a decade and the public exposed, for a similar period of time, to some practices that are causing concern to health authorities all over the world. Nearly every other major country is regularly amending and revising its food laws to take account of constantly changing circumstances and technical advances. Why must the UK lag behind?

During the deliberations leading up to the *Preservatives in Food Regulations*, 1962, the Preservatives Sub-Committee seems to have paid some attention to propositions enunciated in 1924 by the Departmental Committee on the Use of Preservatives and Colouring Matters in Food, namely:-

(i) that it is undesirable to add to articles of food any material not of the nature, substance and quality of food;

(ii) that if for commercial or other reasons the addition is necessary, it should be limited to the minimum required to effect its purpose;

(iii) that if it can be shown that some of these materials are less undesirable than others, preference should be given to the employment of the less undesirable materials.

In the light of the above ideas, that committee then recommended that sulphur dioxide and benzoic acid alone be permitted and these only in a limited number of foods.

The Preservatives Sub-Committee, during their deliberations in the years before 1962 must have kept the second of these sound principles in mind but perhaps not given enough consideration to the first and third. One other requirement, clearly laid down in Section 4 (2) of the *Foods and Drugs Act*, 1955, is that the use of non-nutritive substances be reduced as far as possible and must be shown to be necessary before they can be considered as alternatives or replacements for already approved preservatives. This is not applicable to sorbic acid

TABLE I

	Austria	Belgium	Canada	Denmark	East Germany	Finland	France	Germany	Great Britain	Holland	Italy	Norway	Sweden	Switzerland	USA
Cheese		(x)	x	x		x	x		x		x	x	x		x
Fish preserves	x		x	x	x	x		x		x	x	x	x		x
Delicatessen	x	x	(x)	x	x	x		x		x	x	x	x	x	x
Margarine	x	x	x	x	x	x	x	x		x	x		x		x
Liquid egg	x	x	x		x	x		x		x			x		x
Fruit pulp jams etc.	x	x	x	x	x	x		x		x		x	x	x	x
Soft drinks	x	x	x	x	x	x	(x)	x				x	x	x	x
Wine		x	x	x	x	x	x			x		x	x		x
Dried fruits	x	x	x	x		x	x	x		x	x	x	x	x	x
Pickles	x	x	x	x	x	x	x	x		x		x	x	x	x
Flour confectionery		x	x	x		x			x		x	x		x	x
Bread	(x)	(x)	x			x*		x		(x)	x	x			

( )—only in certain types *—sliced bread

acid is a food preservative akin to certain types of food: it is a lower fatty acid and belongs to that group of fatty acids ingested in large quantities in the normal diet, especially in fats such as butter and coconut oil, from which it differs only in that it contains double bonds and so is unsaturated. The following structural formulae will highlight the similarity

Butyric acid:
$CH_3-CH_2-CH_2-COOH$

Caproic acid:
$CH_3-CH_2-CH_2-CH_2-CH_2-COOH$

Sorbic acid:
$CH_3-CH=CH-CH=CH-COOH$

The metabolism of sorbic acid has been thoroughly investigated in all phases and is identical in human beings and animals. It corresponds in all details to that of any other lower fatty acid. Even with extremely high doses, all of it undergoes metabolism in the organism within a very short time, serving as a source of calories, or is utilised in the biosynthesis of endogenic, higher fatty acids. Since sorbic acid provides the organism with calories, it is not only a preservative but a nutritive substance akin to food and its use thus meets the first principle laid down by the 1924 Departmental Committee. Being a nutritive substance, it falls outside the provisions of Section 4 (2) of the *Foods and Drugs Act* 1955.

Sorbic acid is used all over the world as a preservative for a very wide range of foodstuffs. This is shown in Table I which gives a simplified picture of the position for foods in which it is already permitted by some major countries.

The third principle of the 1924 Departmental Committee seems not to have been closely followed in *Preservatives in Food Regulations* 1962. The desirability or undesirability of the preservatives allowed in these Regulations can be seen easily by looking at the following list of preservatives and their respective LD_{50} values.

	LD_{50} in g/kg
Sorbic acid	about 10·5
Propionic acid	" 9
Salt	" 6
Benzoic acid and parahydroxybenzoates	" 4
Sodium nitrate	" 3
Sulphur dioxide	" 1

An LD_{50} value is expressed in g/kg body weight and is that quantity of a material which, if given to a group of test animals, will kill 50 per cent of them. Thus sorbic acid being tolerated best, is the most desirable whereas sulphur dioxide is the least desirable.

Moreover, the sixth report of the FAO/WHO expert committee on food additives has assessed, at its Session in Geneva, 5-12 June 1961, the acceptable daily intake (ADI) of various preservatives. This committee of the Food and Agriculture Organisation of the United Nations and the World Health Organisation gave two amounts—a lower unconditional ADI maximum and a higher

TABLE II
Per Head
Potential intake of sulphur dioxide in mg/day

Food	oz/wk	g/day	ppm	mg/day
Beer	90·3	365·7	70	25·6
Cabbage, dehydrated			2 500	
Candied peel, etc.			100	
Cider	2·56	10·4	200	2·1
Coffee extract, solid	0·30	1·2	150	0·23
Flavouring emulsions			350	
Flavouring syrups			350	
Flour/biscuit manufacture			200	
Fruit crystallised/glace			100	
Fruit dried	1·03	4·2	2 000	8·4
Fruit/Fruit pulp/manufacturing	0·59	2·4	3 000	7·2
Fruit juices/Welfare orange juice	0·54	2·2	350	0·8
Fruit—other			350	
Gelatin			1 000	
Ginger, dry root			150	
Sacramental grape juice			70	
Horse radish fresh grated			100	
Jam	2·96	12·0	100	1·2
Pectin liquid			250	
Perry			200	
Pickles & Sauces	1·21	5·0	100	0·5
Potatoes raw peeled			50	
Potatoes dehydrated			550	
Sausages or sausage meat	3·72	15·1	450	6·8
Soft drinks (calc. as all diluted)	30·6	124·7	70	8·7
Starches, prepared			100	
Starch, hydrolysed (solid)			70	
Starch, hydrolysed (syrup)			450	
Sugar or sugar syrups	17·68	71·60	70	5·1
Tomato pulp, paste or puree			350	
Vegetable dehydrated (other than cabbage or potato			2 000	
Vinegar			70	
Wine	3·9	15·8	450	7·1
TOTAL				73·73

conditional ADI maximum. Both ADI levels are expressed in mg/kg body-weight and are:

	unconditional max. mg/kg	conditional max. mg/kg
Sorbic acid	12·5	25
Propionates	10·0	20·0
Benzoic acid	5·0	10·0
Sodium nitrate	5·0	10·0
Parahydroxybenzoates	2·0	7·0
Sulphur dioxide	0·35	1·5
Ortho-phenyl phenol	0·2	1·0
Diphenyl	0·05	0·25

In spite of this "degree of desirability" picture, the *Preservatives in Food Regulations* 1962 only permit sorbic acid in 3 foodstuffs (cheese, flour confectionery and marzipan) and 2 food auxiliaries (colouring solutions and silicone emulsions) out of the 52 specified foods whilst the known destroyer of Vitamin B_1, sulphur dioxide, is permitted in 36.

Certain government publications like *Food Facts* and *Household Food Consumption and Expenditure:* 1966 give average consumption figures for 10 of these 36 specified foods and average consumption from 2 more have been obtained from trade sources. Such figures can then be used to calculate some indication of the potential intakes of food additives. For all the food headings, specified and listed in the 1962 Regulations, the picture which emerges for sulphur dioxide is given in Table II.

When it is realised that, due to lack of complete consumption figures, the Table II does not take into consideration the potential intake from 24 out of the above 36 food headings, there can be little wonder at world-wide concern over the level of sulphur dioxide intake.

The figure of 73·53 mg/day for a body weight of 60 kg. (approx. 9½ stone) compares with the acceptable daily sulphur dioxide intake for this same body-weight of conditional maximum 90 mg/day or unconditional maximum 21 mg/day. If one bears in mind that beer—the largest potential sulphur dioxide source—is drunk mainly by the male half of the population, the average potential intake by males might be 120 mg/day, or even more. Moreover, some drink wine as well and one glass of some wines (¼ pint) might contain up to 64 mg sulphur dioxide—*i.e.* three times the unconditional maximum ADI.

For sorbic acid, at the same body-weight the conditional maximum acceptable daily intake is 1 500 mg/day; the unconditional maximum is 750 mg/day. A sorbic acid intake calculation on exactly the same lines as that for sulphur dioxide and including all specified foods for which permission has been granted or, to our knowledge, permission has been requested gives a completely different picture as the following Table III shows.

Appendix III of the 1959 *Food Standards Committee Report on Preservatives in Food* gave the likely actual consumption of sulphur dioxide as unlikely to exceed 20 mg/day. As many of the arguments used to arrive at this reduced sulphur dioxide would equally apply to sorbic acid intakes, the relative positions of the two preservatives would remain little altered.

For example, as in the Food Standards Committee's calculation for likely sulphur dioxide intake, certain factors would similarly reduce the actual sorbic acid intake levels. Sorbic acid would not always be present to the extent of the permitted maximum; only certain types of cheese do really contain any sorbic acid and then nearly always only in the outer layers which are often cut off. Only certain types of bread would contain sorbic acid and, of even greater importance, the bread would only need preserving during a few months of the year; and so on. To be fair, it must also be pointed out that sherry, widely drunk in the UK, has a low sulphur dioxide content so that the SO_2 intake from wine could well be reduced by about 20 per cent.

Obviously sulphur dioxide could not be removed completely from certain foodstuffs where it is required as an antioxidant but the quantities now being used merely to prevent mould growth could safely and with great advantage be replaced by sorbic acid.

TABLE III
Per Head
Potential intake of Sorbic Acid in mg/day

Food	oz/wk	g/day	ppm	mg/day
Cheese	3·36	12·9	1 000	12·9
Preserves	2·96	12·0	500	6
Dried fruits, nuts and nut and fruit products	0·99	4·0	1 000	4
Bread	38·47	156·4	2 000 (on flour wt. i.e. 280/395 of bread)	221·7
Cakes & pastries	4·45	18·0	1 000	18
Pickles & Sauces	1·21	5·0	1 000	5
Fruit juices (incl. welfare orange juice)	0·54	2·2	600	1·3
Wine	3·9	15·8	250	4·0
Fruit or fruit pulp for manufacturing purposes	0·59	2·4	1 500	3·6
Soft drinks	30·8	124·7	160	20·0
TOTAL				292·5

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The Effect of Sorbic Acid on the Exocrine Function of Liver and Pancreas

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In several countries, also in Hungary, sorbic acid and its salts are widely used as preservatives. In food industry these substances are used for the preservation of fruits and vegetables; in each product the permissible level amounts to 0,1 per cent. Besides its perfect preserving effect it has the important property that in the human organism it is metabolised to fatty acid. According to Deuel, Calbert, Anisfeld, Keehan and Blunden [1954 b] sorbic acid gets decomposed to carbon dioxide and water. These substances have been toxicologically investigated by Deuel, Alfin-Slater, Weil and Smith [1954 a] and Ignatev [1965]. The LD_{50} of sorbic acid and its salts is rather high, about 5 — 10 g per kg body weight. In subacute and chronic toxicological tests amounts smaller than 5 — 8 per cent of the dry matter in the forage caused no pathological disorders.

The hygienic assessment of food additives including preservatives needs the greatest possible care, and at the same time more and more sensitive methods. In present investigations, therefore, we wished to apply methods sensitive to different physiological effects. Thus by investigation of the exocrine function of pancreas and liver we followed the effect of acute, subacute and chronic doses of preservatives, administered in amounts considered as ineffective until now.

Methods

In our experiments 168 Wistar rats of the Institute's breed were used.

In the *acute* test on one single occasion sorbic acid has been administered in an amount of about 1,5th of the oral LD_{50}.

In the *subacute* test the rats were given a forage containing 2 per cent sorbic acid (1,6 g/kg body weight) for 12 weeks.

The *chronic* feeding test lasted 22 weeks, and the forage contained 1,5 per cent sorbic acid (1,2 g/kg body weight). The controls were given the usual diet in equal amounts. Besides supervision of the food intake and the weight of the animals, in some of the rats of the chronic group, liver function has been tested by the bromosulphalein retention method [Frommer, 1954]. At the end of the experiment pancreatic juice and bile have been collected by inserting tubes into the ductus choledochus. After measurement of the amount of the juices, their total protein content has been determined. In the pancreatic juice trypsin, chymotrypsin and lipase activity have been estimated by the method of Schön, Rässler and Henning [1961] and the amylase activity by that of Smith and Roe [1949]. In the bile bilirubin [Jendrassik, Cleghorn and Grof (1938)], cholesterol [Rappaport and Eichhorn (1960)], sodium and potassium (by the usual flame spectrophotometry) have been determined. In the chronic experiment the chymotrypsin and lipase activities in the serum have also been investigated. The obtained results concern the juice excreted during 60 minutes.

Results

In Fig. 1 it can be seen that the pancreatic juice increased in all three experiments. In total protein a decrease could be found only in the subacute test. Acute and subacute doses had a strongly decreasing effect on the chymotrypsin activity, whereas in the chronic test this effect was hardly detectable. Lipase activity increased mainly by acute administration and chronic feeding. A decrease in the amylase activity could be only found after subacute administration of sorbic acid.

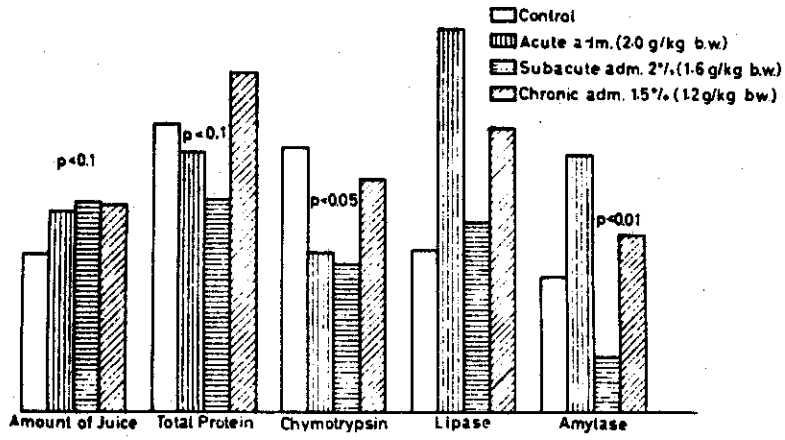

Fig. 1. The effect of sorbic acid administration on the components of pancreatic juice.

In the bile (Fig. 2) the total protein level showed only small differences; acute and subacute doses induced decrease. The cholesterol level decreased only in the acute test. In the chronic test the potassium level of the bile appeared to have an increasing tendency.

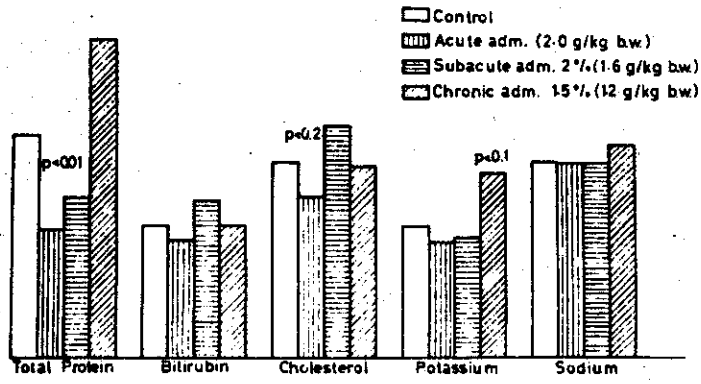

Fig. 2. The effect of sorbic acid administration on the components of bile.

In case of chronic administration of sorbic acid, the chymotrypsin activity in the serum as well as the liver function remained unchanged. Histological findings indicated no pathological disorders in the organs. Besides the effect of the described doses, the influence exerted by smaller doses (subacute 0,25 per cent, chronic 0,5 per cent) has also been investigated. No pathological changes were observed in any case.

Discussion

According to our results differences were more expressed in the exocrine function of the pancreas than in that of the liver. The highest sorbic acid dose—applied in the acute test—resulted in a very accentuated increase of the lipase and amylase activities, and a marked decrease of the chymotrypsin activity. In the subacute test the reduced total protein content and the chymotrypsin and amylase activities indicated an inhibition of the pancreas function. In the chronic test sorbic acid caused not obvious differences. A minor increase in the level of the pancreatic juice and the activity of the lipase indicated a raised challenge to the digestive glands and an increased metabolism of fatty acids. The other findings did not differ from normalcy, showing that an intake of 1,5 per cent sorbic acid in the forage—an amount fifteen times the maximum level permissible for humans—caused no pathological disorders in the exocrine function of pancreas and liver, even in case of continuous intake.

Summary

Acute and subacute administration of sorbic acid and potassium sorbate in amounts considered as ineffective until now, inhibited the exocrine function of the pancreas.

Chronic administration of sorbic acid caused pathological alterations neither in the pancreas nor in the liver function, since by increasing their activity these organs were able to adapt themselves to doses of 1,5 per cent sorbic acid.

The investigation of the exocrine function of pancreas and liver proved to be a method sensitive enough for the assessment of the effect exerted by small amounts of food preservatives.

Literature

Deuel, H. J. jr., Alfin-Slater, R., Weil, C. S. and Smith, H. F.: Sorbic Acid as a Fungistatic Agent for Foods. Food Res. 19: 1 (1954 a).

Deuel, H. J. jr., Calbert, C. E., Anisfeld, L., McKeehan, H. and Blunden, H. D.: Sorbic Acid as a Fungistatic Agent for Foods. Food Res. 19: 13 (1954 b).

Frommer, J.: Effect of PVP on Mouse Liver Function. Proc. Soc. exp. Biol., N. Y. 86: 567 (1954).

Ignatev, A. D.: Kisérleti anyag néhány kémiai konzerválószer kombinált hatásainak egészségügyi jellemzéséhez. (Contributions to the hygienic characterization of the combined effects of some preservatives. Russian.) Voproszi. Pitanija 24: 61 (1965).

Jendrassik, L., Cleghorn, R. A. and Grof, P.: Vereinfachte photometrische Methoden zur Bestimmung des Blutbilirubins. Biochem. Z. 297, 81 (1938).

Rappaport, F. and Eichhorn, F.: Sulfosalicylic Acid as a Substitute for Paratoluene Sulfonic Acid. Clin. Chim. Acta 5: 161 (1960).

Smith, B. W. and Roe, R. J. H.: A Photometric Method for the Determination of α-Amylase in Blood and Urine with the Use of the Starch-Iodine Color. J. biol. Chem. 179: 53 (1949).

Schön, H., Rässler, B. and Henning, N.: Über die Untersuchung der exkretorischen Pankreasfunktion. Klin. Wschr. 39, 217 (1961).

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SORBIC ACID AS A FUNGISTATIC AGENT FOR FOODS. I. HARMLESSNESS OF SORBIC ACID AS A DIETARY COMPONENT[a,b,c]

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It has been discovered by Gooding of The Best Foods Laboratories *(7)* that certain of the α,β-unsaturated fatty acids are good fungistatic agents for foods and food wrappers. Thus, crotonic acid, $CH_3 \cdot CH:CH \cdot COOH$, and its homologues fall into this category. Sorbic acid $CH_3 \cdot CH:CH \cdot CH:CH \cdot COOH$, is particularly well suited as a fungistatic agent for foods. Sorbic acid may be used to prevent mold growth in food products in which sodium benzoate is now employed, *viz.* orange juice, fruit and fruit syrups, pickles, margarine, and candies. In protecting cheeses and cheese products, foods which are very susceptible to mold spoilage, sorbic acid has been found to be markedly superior to sodium benzoate as a fungistatic agent *(17)*. This has also been demonstrated in studies conducted on margarine *(7)*. Phillips and Mundt *(15)* have reported on the advantages of using sorbic acid to inhibit yeast and mold growth while permitting normal fermentation in curing cucumbers.

Over and above the finding that sorbic acid is a superior fungistatic agent, there are certain advantages which would accrue from the use of sorbic acid in place of sodium benzoate. In the first place, a smaller concentration of sorbic acid would be required to protect foods, particularly if protection of the same degree furnished by 0.1% sodium benzoate is desired. Secondly, as will be demonstrated in the second paper of this series *(2)*, the α,β-unsaturated fatty acids are readily metabolizable like any food fatty acid, while benzoic acid cannot be so utilized. Indeed, the α,β unsaturated fatty acids are intermediates in the normal oxidation of the saturated fatty acids. Sorbic acid is metabolizable in exactly the same way as is the natural saturated fatty acid, caproic acid *(2)*, both yielding carbon dioxide and water as end-products. On the other hand, benzoic acid is a strong inhibitor of fatty acid oxidation by liver enzymes *(20)* and must be detoxicated. In man, this occurs by conjugation of the benzoic acid with glycine to yield hippuric acid, $C_6H_5 \cdot CO \cdot NH \cdot CH_2 \cdot COOH$. The latter compound is excreted quantitatively in the urine *(9)*. Some of the benzoic acid

[a]Carried out under a research grant from The Best Foods, Inc.

[b]Paper No. 346 from the Department of Biochemistry and Nutrition, University of Southern California.

[c]The substance of this report comprises part of the material presented as paper 82 at the Boston meeting of the Institute of Food Technologists on June 22-24, 1953.

may be detoxicated with glucuronic acid, derived from glucose, to form phenyl glucuronide *(19)*. In these ways, a compound which resists the oxidative processes of the body is coupled with normal metabolites to yield products which are then readily excreted in the urine. The liver is the site of benzoic acid detoxication, and this fact has actually led to the utilization of hippuric acid excretion as a test of liver function. Individuals with poor liver function will have difficulty in detoxicating benzoic acid.

Before sorbic acid may be used as a fungistatic agent for foods, it would have first to be demonstrated that it is harmless. Two lines of investigations were followed in evaluating this problem. The first involved carrying out conventional toxicity tests, the results of which are presented in this paper. The second involved the metabolic approach to determine the fate of sorbic acid in the animal body.

In this report are summarized the results of studies to determine the LD_{50} of sorbic acid for rats and also to ascertain whether or not this acid exerts a deleterious effect on growth of rats or dogs when fed in amounts many times greater than that required to protect foods from mold spoilage. The tissues of several groups of rats and of the dogs were subjected to histopathological examination at the termination of the test following the 3-month feeding period. An effective part of this toxicity study has dealt with a comparative evaluation of the toxicity of sorbic acid *versus* that of sodium benzoate. In this way, it was possible to assess the toxicity of a new fungistatic agent in relation to another that has been accepted for many years as a fungistatic agent for foods.

In the second report of this series *(2)*, it was possible to relate the metabolism of the unsaturated C_6 fatty acid, sorbic acid, to that of the saturated C_6 fatty acid, caproic acid; the latter is found in butter fat in concentrations of 1.5 to 2.5%. Since it has been demonstrated that the mechanism of fatty acid oxidation in the rat is exactly the same as that in man, the metabolic approach actually constitutes an evaluation of sorbic acid metabolism in the human. The demonstration that the metabolism of sorbic acid and caproic acid is identical in the animal organism is unequivocal proof of the harmlessness of sorbic acid.

The present paper presents the findings obtained in 2 independent investigations, one carried out in the laboratories of the Department of Biochemistry and Nutrition, University of Southern California (Laboratory *A*) and the other at the Mellon Institute of Industrial Research, University of Pittsburgh (Laboratory *B*). Different strains of rats were employed in the 2 laboratories, and the chemicals under test were obtained independently. The dog tests were carried out only in Laboratory *A*.

EXPERIMENTAL AND RESULTS

The experiments of Laboratory *A* were carried out on rats of the University of Southern California strain while those employed in the tests by Laboratory *B* were animals of the Sherman strain selected from rats raised in its own laboratory.

The identity of the samples with sorbic acid[a] was established by determination of

[a]The sorbic acid employed in the tests in Laboratory *A* was either obtained directly from the Carbide and Carbon Chemicals Company, New York, after June 1951 (S-93868), or from a sample supplied by The Best Foods who had purchased it from the above source (S-33568).

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melting point and equivalent weight as well as by general crystalline form and solubility tests carried out in two cases in the Southern California laboratories. Spectrophotometric absorption curves obtained in the laboratory of The Best Foods, Inc. indicated that the sorbic acid was better than 99% pure on a moisture-free basis. All the sorbic acid used by Laboratory *B* was obtained from a 10-pound sample of commercial grade sorbic acid,* labeled "Drum No. 460-R." It was received on July 4, 1950. U.S.P. sodium benzoate (Merck) was used in the toxicity tests carried out in both laboratories. The purity of the caproic acid† used in the dog tests was ascertained by the determination of equivalent weight, refractive index, density, melting point, and boiling point tests.

Acute toxicity tests. In the toxicity tests carried out in Laboratory *A*, male rats, weighing 212 to 430 grams, and female rats, weighing 163 to 267 grams were used. The method of testing was similar to that employed earlier *(3)*. This involved feeding an appropriate dose of the test substance by stomach tube to animals previously fasted for 18 hours. The animals were kept in separate cages and were provided with stock diet and water. The tests were considered positive if the animals died within 5 days. This interval was chosen since by this time practically all animals which had survived were again gaining weight, and were in satisfactory nutritional condition. Toxicity studies in Laboratory *A* were made only with the sodium salts of sorbic and benzoic acid. A total of 182 rats were used for the tests with sodium sorbate, and a total of 70 rats for those with sodium benzoate by Laboratory *A*. In the case of Laboratory *B*, acute toxicities were determined of both free sorbic acid and sodium sorbate as well as of sodium benzoate. Sorbic acid was fed as a suspension which was prepared as follows: The acid was ground fine in a mortar moistened with a few drops of 1% "Tergitol" Penetrant 7, and 0.25% agar solution was added to give a suspension containing 0.20 g. sorbic acid per ml. The suspension did not separate during 15 minutes' standing; gentle shaking resuspended it after the suspension was allowed to stand over a prolonged period. Sodium sorbate and sodium benzoate were fed in 10% aqueous solution. In the series of tests carried out by Laboratory *B*, 90 to 120 g. unfasted rats were used. There were 5 males and 5 females in each group and the animals were observed for 14 days after the dosing. The data on the toxicity tests are summarized in Table 1.

TABLE 1

Average LD_{50} of sorbic acid when fed as free acid or sodium salt and of sodium benzoate in rats

Category	Laboratory *A*	Laboratory *B*
LD_{50} for sorbic acid	.....	10.50±1.96 g./kg. (9.17-12.03)
LD_{50} for sodium sorbate (calculated as sorbic acid)	4.0 g./kg.	5.94 g./kg. (5.65-6.23)
LD_{50} for sodium benzoate (calculated as benzoic acid)	2.1 g./kg. (1.7-2.5)	3.45 g./kg. (3.15-3.74)
LD_{50} (sodium sorbate) : LD_{50} (sodium benzoate)	1.90	1.72

*Average of 4.3 for males and 3.65 for females.

The LD_{50} values for free sorbic acid are considerably higher than those for the more soluble sodium sorbate. This indicates that sorbic acid when fed as such is less rapidly absorbed than is sodium sorbate. The average LD_{50} figure for sodium sorbate was found to be 4.0 g./kg. by Laboratory *A* and 5.94 g./kg. by Laboratory *B*. The variation is to be expected since different sized rats were employed; moreover, in one case, the material was given to fasted rats and in the other case to rats which had partaken

*Obtained from the South Charleston plant of Carbide and Carbon Chemicals Company.

†Obtained from the Matheson Co., East Rutherford, N. J.

of their food. On the other hand, the LD_{50} of sodium sorbate was almost twice that of sodium benzoate. The comparative ratios of LD_{50} of sorbate to benzoate reported by the two laboratories are quite consistent, namely 1.90 and 1.72.

Feeding tests on rats. It has been suggested *(8)* that fatty acids with conjugated double bonds might function in the animal organism as antimetabolites of the essential fatty acids. Thus, it is conceivable that elaeostearic acid, 9,11,13-octadecatrienoic acid, might act as an antagonist to linolenic acid, 9,12,15-octadecatrienoic acid. Moreover, sorbic acid, which has 2 conjugated double bonds, might be expected if the above hypothesis were true, to exert a similar action toward the essential dienoic acid, linoleic acid, which is 9,12-octadecadienoic acid.

However, there is considerable evidence that elaeostearic acid can be metabolized. Thus, Miller and Burr *(11)* reported that the trienoic acids in tung oil, which consists of approximately 80% of elaeostearic acid, are converted to diene acids which are gradually metabolized. Reiser *(16)* reports a similar metabolism of elaeostearic acids in hens with its deposition in the egg, while Fillerup and Mead *(5)* have demonstrated that methyl-β-elaeostearate is absorbed at the same rate as methyl oleate. In none of these tests was there any evidence of toxicity by the conjugated trienoic acid. Although these tests do not give information as to any possible deleterious action of elaeostearic acid over long periods of time, they do indicate that no immediate toxicity obtains and likewise that a trienoic acid can be converted to a dienoic acid which is eventually metabolized. These findings in themselves cast doubt upon the possibility that fatty acids with double bonds in conjugated position are harmful.

In order to test whether or not sorbic acid or elaeostearic acid acts as an antimetabolite toward the essential fatty acids, these metabolites were incorporated at several levels in the fat-free diet employed earlier by Anisfeld *et al. (1)* to produce essential fatty acid deficiency. Rats receiving the fat-free diet without added sorbic acid or tung oil provided a control group. At the same time, records of food consumption and body weight were kept, from which data it is possible to calculate the efficiency of food utilization. The composition of the diets is recorded in Table 2, while the data on growth on the several diets are summarized in Table 3.

There is no evidence on the basis of the data recorded in Table 3 that the incorporation of sorbic acid or elaeostearic acid into a fat-free diet accelerates the rate of disappearance of the unsaturated fatty acids in the tissues and so shortens the depletion time for essential fatty acids. In fact, at 11 weeks, when depletion had occurred in the control group, there were no symptoms indicating that essential fatty acid depletion had occurred in the groups receiving sorbic acid or tung oil. Although Group 35c might appear to be depleted, the loss in weight was shown to be due to the failure of these rats to eat. When these rats were placed on the fat-free control diet, they continued to gain weight until the 19th week when the experiment was terminated.

The gains-in-weight of the rats receiving sorbic acid are identical, both at 6 weeks and at 11 weeks, with those of the rats on the control diet. Thus, after 11 weeks on the several diets, the average gains-in-weight were 137.0 ± 7.2 g., 152.0 ± 8.4 g., and 146.9 ± 6.3 g. for the rats receiving 0.5, 1.0, and 5.0% of sorbic acid respectively in the diets, as compared with a control value of 151.9 ± 4.3 g. Inasmuch as there was no relationship in weight gain to the sorbic acid content of the diet, the figures at 11 weeks are considered to be the same; the variations noted are due to the imprecisions of such biological tests. Actually, the values for the animals on the lowest (0.5%) sorbic acid diet may be regarded as constituting another control group.

The efficiencies with which the diets are utilized show no statistically significant differences. The average figures for efficiency ratio would seem to indicate less efficient utilization of the sorbic acid diets, if one were to disregard variability in responses of the animals. However, here also there is no tendency for the efficiency of food utilization (including the calories from sorbic acid) to decrease with an increasing content of sorbic acid in the diet. One could justifiably consider Group 38a which received 0.5% sorbic acid in the diet as another control group in evaluating the findings with animals on the 5% sorbic acid diet. On the basis of such a comparison, sorbic acid would necessarily be regarded as a source of calories in the diet. However, because of the small increment of calories which is contributed by sorbic acid, because of the *ad libitum* feeding, and because the growth response in relation to calories is on the upper plateau of the curve, the findings do not unequivocally prove that sorbic acid can

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TABLE 2

Composition of the diets used in the growth tests with sorbic acid and with tung oil

LABORATORY A

Food component	Fat-free diet (39)	Sorbic acid diets			Tung oil diets		
		38a	38b	38c	35a	35b	35c
	%	%	%	%	%	%	%
Casein, vitamin test[a]	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Sucrose	71.62	70.75	69.88	62.92	71.02	70.42	65.62
Cellulose[b]	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Salt mixture[c]	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Sorbic acid[d]	....	0.50	1.0	5.0	....	....	....
Tung oil[e]	....	....	....	....	0.6	1.2	6.0
$NaHCO_3$[f]	....	0.37	0.74	3.70	....	....	....
Water-soluble vitamins[g]	0.38	0.38	0.38	0.38	0.38	0.38	0.38
Fat-soluble vitamins[h]	....	....	....	....	....	....	....
Caloric value per 100 g.[i]	368.4	368.3	368.2	364.8	....	....	....

[a] General Biochemicals, Chagrin Falls, Ohio.
[b] Solka Floc, which is a cellulose flour.
[c] Salt mixture of Osborne-Mendel *(13)*.
[d] Purchased from Union Carbide and Chemicals Company.
[e] The sample of tung oil was supplied by The Best Foods, Inc. Spectrophotometric analysis gave a value of 87.8% elaeostearic acid, expressed on the total fatty acid content.
[f] Chemical equivalent of sorbic acid added.
[g] The water-soluble vitamins containing menadione were added in the following amounts per kg. of diet:

Thiamine	36 mg.	Folic acid	10 mg.
Riboflavin	72 mg.	Niacine	60 mg.
Pyridoxine	27 mg.	PABA	1,000 mg.
Calcium pantothenate	67 mg.	Inositol	500 mg.
Menadione	5 mg.	Choline	2,000 mg.
Biotin	1 mg.	Vitamin B_{12} (added in 95% ethyl alcohol)	0.01 mg.

The total is 3,778.01 mg./100 g. or 0.38%. Thiamine, riboflavin, pyridoxine, calcium pantothenate, niacin, choline, and vitamin B_{12} were kindly furnished by Merck and Co., Inc.; biotin was contributed by Hoffman-LaRoche, Inc.; and folic acid was a gift of the Lederle Laboratories.
[h] The fat-soluble vitamins A, D, and E were given orally once weekly. A total of 4 drops of the following mixture was given to each rat: 2.2 g. Nopco oil (800,000 U.S.P. units vitamin A and 80,000 U.S.P. units vitamin D per g.) and 51.5 g. of mixed tocopherols (340 mg. α-tocopherol per g.) made up to 200 ml. with propylene glycol.
[i] Based on a caloric value of 6.63 Cal./g. for sorbic acid *(12)*.

be utilized for growth as a source of calories. A more precise and direct approach is described in the second paper of this series *(3)*. In any case, the results reported here definitely indicate that sorbic acid does not interfere with the utilization of other foodstuffs for maintenance and growth.

Subacute toxicity studies of sorbic and benzoic acids on rats. The subacute toxicity tests were performed in Laboratory *B* in much the same way as the feeding tests described above except that the animals were sacrificed after 90 days on the diets and their tissues subjected to pathological examination.

The basal diet employed in these studies was a modified Food Research Laboratory diet 2C *(6)* consisting of dried whole milk, ground whole wheat, salt, yeast, and liver extract. It was freely available in McCollum-type food cups. Sorbic acid or sodium benzoate was fed at levels of 1, 2, 4, and 8% of the diet. One group subsisted on the diet without any additions, and one group on a 0.5% sorbic acid diet. Each group consisted of 5 male and 5 female rats picked at random from young rats raised in the Mellon Institute laboratory.

Tables 4 and 5 give the significant data for the groups receiving sorbic acid or sodium benzoate respectively. The level of statistical significance is indicated by the determination of the value for "p" for the "t" test for differences of each mean from that of the specific control group. When "p" is 0.05, the chance of the average being different from the control is one in 20. Only when the p-figures are 0.05 or less, are the results considered significant.

The growth tests with sorbic acid reported in Table 4 in Laboratory *B* confirm in every respect those carried out in Laboratory *A*, which are summarized in Table 3. Thus, the food consumption and gain-in-weight of the rats over the 90-day experi-

TABLE 3

The body weights of male rats receiving a fat-free diet or ones containing different levels of sorbic acid or tung oil as well as calculations of efficiency of utilization of the sorbic acid diets

LABORATORY A

Category	Groups receiving following diets						
	(Fat-free) 39	Sorbic acid tests			Tung oil tests		
		38a	38b	38c	35a	35b	35c
Number of rats	20	10	10	10	10	10	10
Test material in diet, %	....	0.5	1.0	5.0	0.6	1.2	6.0
Average body weight, g.							
Start	36	34	34	36	35	34	36
After 3 weeks	96	98	100	98	99	101	95
After 6 weeks	154	146	155	154	158	165	157
After 9 weeks	186	164	172	171	189	188	179
After 11 weeks	189	171	186	183	212	201	172
After 19 weeks*	217	206	221	233	241	254	263
Average gain-in-weight, g.[b]							
After 6 weeks	117.8±3.4	112.0±6.9	120.4±8.2	118.2±3.6	....	....	....
After 11 weeks	151.9±4.3	137.0±7.2	152.0±8.4	146.9±6.3	....	....	....
Average food consumed, g.							
After 6 weeks	436.6	455.5	485.9	497.4	....	....	....
After 11 weeks	949.0	907.2	966.7	987.9	....	....	....
Average food efficiency[b, c]							
After 6 weeks	7.30±0.17	6.67±0.22	6.69±0.26	6.56±0.19	....	....	....
After 11 weeks	4.33±0.12	4.19±0.22	4.25±0.13	4.08±0.17	....	....	....
Average total sorbic acid eaten, g.	0	4.53	9.67	49.40	....	....	....

* Rats received fat-free diet starting on 12th week.

[b] Including Standard Error of Mean calculated from the formula $\sqrt{\Sigma d^2/n-1}/\sqrt{n}$ where "n" is the number of observations and "d" is the deviation from the mean.

[c] Calculated by dividing the gain in body weight × 100 by the calories consumed.

TABLE 4

The results of the 90-day feeding tests on rats receiving diets containing different levels of sorbic acids or a sorbic acid-free control diet

LABORATORY B

Category	Sorbic acid-free control diet	Diets containing sorbic acid in %				
		0.5	1.0	2.0	4.0	8.0
Sorbic acid dosage, g./kg./day	0.0	0.32	0.63	1.26	2.48	5.06
Diet consumed, g./rat/day	11.97	12.57	12.62	11.98	12.08	12.36
p for t—test	....	0.65	0.55	0.99	0.93	0.72
Av. weight gain of surviving rats, g.	156.3	164.4	169.0	161.8	190.1	158.6
p for t—test	....	0.76	0.60	0.84	0.29	0.94
Liver weight, % body weight	3.17	3.20	3.27	3.25	3.52	3.54*
p for t—test	....	0.83	0.52	0.51	0.06	0.04
Kidney weight, % body weight	0.64	0.63	0.64	0.62	0.65	0.65
p for t—test	....	0.65	1.00	0.24	0.73	0.65
Uninfected rats**	9	9	9	9	7	7
Toxic deaths	0	0	0	0	0	0
Sets of rat tissue with any pathology	0	0	0	0	0	0

* Results statistically significant when compared with control.

** Tissues examined from all these rats.

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LABORATORY A

diets

Tung oil tests		
35a	35b	35c
10	10	10
0.6	1.2	6.0
35	34	36
99	101	95
158	165	157
189	188	179
212	201	172
241	254	263
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LABORATORY B

ing sorbic acid in %

2.0	4.0	8.0
1.26	2.48	5.06
11.98	12.08	12.36
0.99	0.93	0.72
161.8	190.1	158.6
0.84	0.29	0.94
3.25	3.52	3.54*
0.51	0.06	0.04
0.62	0.65	0.65
0.24	0.73	0.65
9	7	7
0	0	0
0	0	0

mental period in the case of all groups receiving sorbic acid (including at an 8% level) are not different from those of the control group. Moreover, all tissues which were examined of all the rats on the several sorbic acid regimens were normal. There was a slight increase in liver weights as percentages of the body weights in the group receiving 8% sorbic acid (average liver weight being 3.54% of body weight) when compared with those on the control diet (3.17% of body weight). Although this figure for the 8% sorbic acid group is significantly higher than that of the control ($p = 0.04$, barely over the usually accepted level of significance), even in this case it must be considered as representing merely an extremely mild enlargement of normal liver; no micropathology of the liver was evident at any level of sorbic acid fed. The percentage of body

TABLE 5

The results of the 90-day feeding tests on rats receiving diets containing different levels of sodium benzoate or a sodium benzoate-free control diet

LABORATORY B

Category	Sodium benzoate-free control diet	Diets containing sodium benzoate in %			
		1.0	2.0	4.0	8.0
Sodium benzoate dosage, g./kg./day	0.0	0.64	1.32	2.62	6.29
Diet consumed, g./rat/day	11.97	12.69	12.63	12.30	11.98
p for t—test	....	0.54	0.62	0.75	0.99
Average weight gain of surviving rats, g.	156.3	170.7	160.5	150.0	100.2
p for t—test	....	0.58	0.86	0.82	0.06
Liver weight, % body weight	3.17	3.01	2.97	3.12	4.46*
p for t—test	....	0.26	0.26	0.89	0.0005
Kidney weight, % body weight	0.64	0.64	0.65	0.70	0.90*
p for t—test	....	1.00	0.65	0.06	0.0001
Uninfected rats	9	9	10	8	8
Toxic deaths	0	0	0	0	4
Average number days to death	....	....	....	....	13.0
Sets of tissues examined from uninfected rats	9	9	10	8	7
Sets with major pathology	0	0	0	0	1
Sets with any pathology	0	1	1	1	7

* Results statistically significant as compared with control.

weight represented by the weight of the liver has been reported in other studies *(4)* to be as high as 5.34% after a choline-free diet containing butter fat which was administered for only 16 days, in contrast to a value of 3.17% when choline was added to the regimen. In Laboratory *B*, no change in kidney weight was observed, as related to the sorbic acid intake and no histopathological changes in that organ. It may be concluded that sorbic acid when incorporated in the diet at a level as high as 4% has no harmful effect on rats; at 8%, it is highly doubtful that any toxicity occurs within 90 days.

The results obtained in the rats receiving sodium benzoate are in striking contrast to those noted in the sorbic acid tests. When this substance was present in the diet at the 8% level, the gain-in-weight of the *survivors* in the group over the 90-day period was only two-thirds of that of the control rats on an identical food intake. Statistical analysis of the growth responses indicated that this was just below the usually accepted level of significance ($p = 0.06$). This interpretation of a non-significant difference does not mean that a real difference does not exist; the limited number of survivors, 4 out of the original group of 8, resulted in the evaluation of such a limited number of responses that differences would have to be enormous to be significant. Furthermore, the weights of both the livers and kidneys of the rats in this group were significantly higher ($p = 0.0005$ and 0.0001, respectively) than were those of the control group. The weights of these organs were 140% of those of the control rats. Four rats died without infections during the study, and one is forced to conclude that death was caused by the agents fed. Moreover, frequent pathological lesions were noted in the

livers and kidneys of the group which received 8% of sodium benzoate, although they were seldom severe.

At the 3 lower levels of benzoate feeding (4, 2, and 1%) one liver in each case was found with light cloudy swelling among the uninfected survivors of the several groups. Although this degree of pathological lesions was not found in the specific controls of the study, it is not an infrequent finding in control animals and probably should be considered as an artifact. If one accepts such an assumption, one is justified in concluding that 4% of sodium benzoate in the diet is harmless under the conditions of the test.

On the basis of these subacute toxicity tests, one may state that sorbic acid and sodium benzoate when fed at a level of 4% in the diet have no harmful effect on rats in a 90-day feeding study. Sorbic acid at 8% is probably harmless while sodium benzoate at an 8% level is definitely toxic.

Subacute toxicity tests of sorbic and caproic acids on dogs. The tests on dogs were designed to determine whether or not sorbic acid exerts any harmful effects when fed as the free acid to puppies over a 3-month period following weaning. Two sets of controls were carried out, *viz.*, one in which the dogs received a comparable dose of caproic acid to that of sorbic acid and the second in which the dogs received only the basal diet.

Nine puppies from 3 litters were purchased commercially. The dogs from each litter were distributed between the 3 dietary regimens. In the case of litter 2, which comprised 4 dogs, 2 were placed on the sorbic acid regimen. The dogs ate all 3 diets with gusto and only on several occasions were the diets not completely consumed. The animals were kept in separate cages for the duration of the test except that they were exercised in a large yard for one-hour periods each morning and afternoon. The dogs were fed once daily. Daily records were kept of their body weights. After the animals had been on several diets for 90 days, they were sacrificed, blood samples were obtained for hemoglobin determinations and the following organs were removed: liver, kidney, heart, lung, pancreas, stomach, small intestine, spleen, adrenal glands, thyroid gland, parathyroid gland, gonad, striated muscle and skin. The tissues and/or organs from each animal were placed in 10% formalin fixative in glass jars and submitted to the pathologist* for histopathological examination with a code number known only to the laboratory worker submitting the samples.

The composition of the several diets is given in Table 6. The presence of sorbic or caproic acids in the diets was confirmed by the analyses carried out in the laboratory of The Best Foods, Inc. of samples forwarded to them; these analyses are recorded in Table 7. Table 8 gives the data on the food consumption and weight gains in the dogs.

The animals seemed uniformly healthy, vigorous, and frisky during the experimental period except for a short interval when Dog 36 showed the presence of coccidiosis in the stools. The infection had been present in dogs previously purchased which had been discarded before being used for experiment. None of the other dogs employed in the present study became infected and Dog 36 made a quick recovery. At no time during the experimental period were there any additions or subtractions from the diet.

With the exception of Dog 36 which had lost considerable weight during the period when he had coccidiosis, the gain-in-weight of the animals in any one litter on the several dietary regimens was fairly uniform. For example, in litter II, both dogs on the sorbic diet gained more than the caproate control but slightly less than the dog on the control diet. In the other 2 litters, the dogs receiving the caproate diet did as well as those on the control diet.

The level of hemoglobin at the termination of the tests was satisfactory in all cases. The values averaged as follows: control diet, 11.4% (3 dogs); caproic acid diet, 13.6% (3 dogs); and sorbic acid, 12.2% (2 dogs). Although some minor pathological changes were noted in the tissues, no alteration was found to occur repeatedly, with any significance, in any one group. It was therefore concluded that sorbic acid in an amount of 4% (5% on a moisture-free basis) in the diet was harmless to dogs. This conclusion was based on the fact that the animals remained in excellent condition throughout, by the lack of any specific abnormalities which could be detected on gross examination of

*We are indebted to Dr. H. Russell Fisher, Professor of Pathology, University of Southern California School of Medicine, for the histopathological examinations.

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TABLE 6

Composition of the diets used in the growth tests of dogs with basal diet alone or with basal diet containing sorbic or caproic acid [a]

LABORATORY A

Food component	Basal diet (1)	Basal diet containing caproic acid (2)	Basal diet containing sorbic acid (3)
	%	%	%
Cheddar cheese (mild) [b]	50.0	50.0	50.0
Margarine fat	2.0	0.0	0.0
Cod-liver oil	5.0	5.0	5.0
Sucrose	18.5	16.5	16.5
Dextrin	15.0	15.0	15.0
Cellu flour [c]	2.0	2.0	2.0
Salt mixture [d]	1.5	1.5	1.5
Dried brewer's yeast [e]	5.0	5.0	5.0
Liver extract [f]	1.0	1.0	1.0
Caproic acid	0.0	4.0	0.0
Sorbic acid	0.0	0.0	4.0

[a] The control basal diet contains 20% of moisture. Diets 2 and 3, containing caproic and sorbic acid, respectively, involve an iso-caloric substitution. The diets are prepared as follows: To the cheese, melted to a flowable mass, are added without further heating the cod-liver oil and the melted margarine fat, caproic acid, or sorbic acid, as the case may be. The mixed dry ingredients are then added, and the blended mixture stored in the refrigerator. The chilled product was broken into small bite-size granules prior to feeding. Diets were kept at 45°F. for not longer than 2 weeks.

[b] Purchased in 25-lb. wheels from the Challenge Dairies, Los Angeles, Calif.

[c] Solka Floc which is a cellulose flour.

[d] Wesson modification *(18)* of salt mixture of Osborne-Mendel *(13)*.

[e] Anheuser-Busch, Strain G.

[f] Wilson, 1:20 liver concentrate.

TABLE 7

The analysis of samples of diets used in dog experiments for sorbic acid and total fat [a]

LABORATORY A

Category	Fresh (F) or week-old diet (O)	Basal diet (1)	Basal diet containing caproic acid (2)	Basal diet containing sorbic acid (3)
pH of 10% suspension in distilled water	F	5.9	4.8	5.0
	O	5.9	4.8	5.0
Sorbic acid, % [b]	F	....	....	4.2
	O	....	....	4.4
Fat, %	F	22.8	24.7	24.8
Analysis of fat:				
Acid value [c]	F	0.4	130.4	125.3
	O	0.7	130.7	125.7
Peroxide value [d]	F	5.9	6.0	6.1
	O	13.2	13.4	16.5
Thiobarbituric acid reaction, [e] ($E^{1\%}_{1cm.}$ at 535 mμ)	F	0.086	0.091	0.092
	O	0.108	0.127	0.126

[a] Analyses conducted in the laboratory of The Best Foods, Inc. The diets were analyzed as received and after one week at 75°F.

[b] Analysis by spectrophotometric method described in *(10)*.

[c] Mgs. KOH per g. sample.

[d] Milliequivalents per kg.

[e] A measure of aldehydic oxidation products (fat basis) which react with 2-thiobarbituric acid to give a pink color with maximum absorbency at 535 mμ under conditions of the test *(14)*.

TABLE 8

Summary of feeding tests on dogs

LABORATORY A

Litter and dog number	Description of litter	Dietary regimen[a]	Sex	Duration of feeding test	Weight in grams			Hemoglobin at end
					Start	End	Gain	
				days				%
I, 34	Mixed cocker and terrier	1	F	91	1800	5100	3300	14.0
57		2	M	91	2425	5870	3445	13.8
36		3	M	89	2300	4430	2130	....[b]
II, 50	Half cocker	1	M	88	1407	4360	2853	10.3
38		2	M	88	1365	3480	2015	14.0
33		3	F	88	1700	4370	2670	13.0
59		3	M	88	1680	4000	2320	11.5
III, 37	Half cocker	1	M	90	2045	5800	3755	10.0
2		2	M	90	2145	6300	4155	13.0

[a] Diet 1 is control diet; Diet 2 contains 4% caproic acid (wet basis); and Diet 3 contains 4% sorbic acid (wet basis).

[b] Dog 1c (No. 36) was run over and killed on 89th day. Animal had had coccidiosis from 20th to 25th day but had recovered.

the organs of the animals when they were sacrificed at the end of the feeding experiments, and by the report of the histopathological examination of the tissues which showed no abnormal conditions.

SUMMARY

1. The results of the present experiments indicate that sorbic acid is harmless to rats and to dogs when incorporated in the diets to the extent of 5% (moisture-free basis). This conclusion is based on the following experimental findings:

a. In tests with 2 different strains of rats conducted in 2 separate laboratories, the gain-in-weight of rats receiving diets containing up to 8% of sorbic acid was similar to that of rats on the basal diet free of sorbic acid. Similar results were obtained in one series of tests involving supplementation of a fat-free diet with 5% sorbic acid.

b. There was no evidence of abnormality in liver or kidney weights or of pathological conditions in other tissues of the rats receiving 4% of sorbate. Rats on the 8% sorbic acid diet exhibited a slight but statistically significant increase in liver weight, expressed as percentage of body weight, but this represented merely an extremely mild enlargement of liver without micropathology.

c. Sorbic acid does not act as an antimetabolite for essential fatty acids in rats. It was likewise found that elaeostearic acid did not act as an antagonist to the essential acids.

d. The incorporation of sorbic acid into the diet did not decrease the utilization of the foodstuffs fed concomitantly, as determined from the efficiency ratio (gain-in-weight $\times$ 100) : calories consumed. Indeed, the present findings would seem to indicate that sorbic acid is utilizable by the animal organism as a source of calories. The results of metabolic studies to be reported have confirmed this interpretation.

e. In tests with dogs, similar response was obtained when the animals received a 50% Cheddar cheese diet with other food components alone, or

ogs

LABORATORY A

Weight in grams			Hemoglobin at end
rt	End	Gain	
			%
00	5100	3300	14.0
25	5870	3445	13.8
00	4430	2130	....
07	4360	2853	10.3
35	3480	2015	14.0
00	4370	2070	13.0
80	4000	2320	11.5
45	5800	3755	10.0
45	6300	4155	13.0

wet basis); and Diet 3 contains

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the same diet with 4% sorbic acid or 4% caproic acid incorporated as the free acid. A histopathological examination of tissues at the termination of the tests failed to indicate any pathological lesions attributable to the experimental diets.

2. The results of the present experiments indicate also that sorbic acid is far less toxic than sodium benzoate. This conclusion is based upon the following experimental findings:

a. Sorbic acid has been shown in 2 independent laboratories to have a relatively high LD_{50}. When fed as a free sorbic acid to rats, in one laboratory, the LD_{50} was found to be 10.50 g./kg. When fed as sodium sorbate to rats having access to food, the LD_{50} was found to be 5.94 g./kg. (calculated as sorbic acid); on the other hand, when fed to fasted rats, the LD_{50} was found to be 4.3 g./kg. for male rats and 3.65 g./kg. for female rats.

b. Sodium benzoate was found to have an LD^{50} of 3.45 g./kg. in one laboratory with rats having access to food, and 2.1 g./kg. in another laboratory with fasted rats. The ratios of LD_{50} (sodium sorbate) : LD_{50} (sodium benzoate) based on calculations as the free acids, were found to be 1.72 and 1.90 in the two laboratories.

c. Unlike sorbic acid, sodium benzoate was definitely toxic to rats when fed at a level of 8% of the diet. Growth and survival were adversely affected and histopathological changes in liver and kidney occurred.

LITERATURE CITED

1. Anisfeld, L., Greenberg, S. M., and Deuel, H. J., Jr. The lack of correlation between the growth response of the fat-depleted rat to essential fatty acids and the tocopherol of the diet. *J. Nutrition*, 45, 599 (1951).
2. Deuel, H. J. Jr., Calbert, C. E., Anisfeld, L., McKeehan, H., and Blunden, H. Sorbic acid as a fungistatic agent in foods. II. Metabolism of α,β-unsaturated fatty acids with emphasis on sorbic acid. *Food Research*, 19, 13 (1954).
3. Deuel, H. J., Jr., Greenberg, S. M., Calbert, C. E., Baker, R., and Fisher, H. R. Toxicological studies on isopropyl and stearyl citrates. *Food Research*, 16, 258 (1951).
4. Deuel, H. J., Jr., Murray, S., Hallman, L. F., and Tyler, D. B. Studies on ketosis. XII. The effect of choline on the ketonuria of rats following a high fat diet. *J. Biol. Chem.*, 120, 277 (1937).
5. Fillerup, D. L., and Mead, J. R. Absorption and distribution studies using cleostearic acid. U. S. Atomic Energy Commission, U.C.L.A. *Report No. 148*, August 7, 1951.
6. Hawk, P. B., Oser, B. L., and Summerson, W. H. *Practical Physiological Chemistry*. 12th ed., 1947, Blakiston, Philadelphia, p. 1272.
7. Gooding, C. M. Process of inhibiting growth of molds. U. S. Patent 2,379,294 (1945).
8. Gould Lectures, *Lipids*, University of Rochester, September, 1950.
9. Knoop, F. Der Abbau aromatischer Fettsäuren im Tierkörper. *Beitr. chem. Physiol. u. Pathol.*, 6, 150 (1904).
10. Melnick, D., and Luckman, F. H. Sorbic acid as a fungistatic agent for foods. III. Spectrophotometric determination of sorbic acid in cheese and in cheese wrappers. *Food Research*, 19, 20 (1954).
11. Miller, E. S., and Burr, G. O. Photoelectric spectrophotometry applied to studies in fat metabolism. *Proc. Soc. Exptl. Biol. Med.*, 36, 726 (1937).
12. National Research Council, *International Critical Tables*, Vol. V. 1929, McGraw-Hill, New York, p. 165.
13. Osborne, T. B., and Mendel, L. B. The nutritive value of the wheat kernel and its milling products. *J. Biol. Chem.*, 37, 557 (1919).

14. PATTON, S., AND KURTZ, G. W. 2-Thiobarbituric acid as a reagent for detecting milk fat oxidation. *J. Dairy Sci.*, 34, 669 (1951).
15. PHILLIPS, G. F., AND MUNDT, J. O. Sorbic acid as an inhibitor of scum yeast in cucumber fermentations. *Food Technol.*, 4, 291 (1950).
16. REISER, R. The biochemical conversions of conjugated dienoic and trienoic fatty acid. *Arch. Biochem.*, 32, 113 (1951).
17. SMITH, D. P., AND ROLLIN, N. Sorbic acid as a fungistatic agent for foods. VII. Effectiveness of sorbic acid in protecting cheese. *Food Research*, 19, 59 (1954).
18. WESSON, L. G. A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. *Science*, 75, 339 (1932).
19. WEST, E. S., AND TODD, W. R. *Textbook of Biochemistry*. 1951, Macmillan, New York, p. 530.
20. WITTER, R. F., NEWCOMB, E. H., AND STOTZ, E. The oxidation of hexanoic acid and derivatives by liver tissue *in vitro*. *J. Biol. Chem.*, 185, 537 (1950).

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SORBIC ACID AS A FUNGISTATIC AGENT FOR FOODS. II. METABOLISM OF α,β-UNSATURATED FATTY ACIDS WITH EMPHASIS ON SORBIC ACID[a,b,c]

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In the first paper of this series *(7)*, it was reported that sorbic acid, $CH_3 \cdot CH:CH \cdot CH:CH \cdot COOH$, when administered as the free acid or as the sodium salt, has a lower toxicity than sodium benzoate. There was also some evidence that sorbic acid might be metabolized by the animal and used for growth as a source of calories, although the experiments were not carried out with sufficient precision to establish this point unequivocally.

However, if it could be demonstrated that sorbic acid is metabolized in the same manner as the normally-occurring saturated fatty acids, then one would have proof of its complete utilization for the production of energy. The best way to approach the solution to this problem is to determine to what extent the acid in question contributes to the development and excretion of ketone bodies in comparison with other fatty acids. A ketonuria does not occur normally when adequate carbohydrate is present in the diet. However, in the absence of the latter foodstuff, all fatty acids of even number carbon atoms are converted to ketone bodies which accumulate and are then excreted as such in the urine. These ketone bodies represent intermediates in the fatty acid oxidation which under the influence of carbohydrates are completely oxidized to carbon dioxide and water.

There are several experimental reports which suggest that the metabolism of sorbic acid and of the naturally-occurring hexanoic acid, caproic acid ($CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot COOH$), is similar. The first hint of the identity in metabolic pathways of sorbic and caproic acids was the work of Cohen *(5)*. He found that sorbic acid is strongly ketogenic and has a Q_{AcOAc} value of the same order as caproic acid. Witter, Newcomb, and Stotz *(16)* reported that sorbic acid, 2-hexenoic acid ($CH_3 \cdot CH_2 \cdot CH_2 \cdot CH:CH \cdot COOH$), as well as caproic acid, are quantitatively transformed to acetone bodies, since all 3 acids, on a molar basis, yielded 1.5 moles of acetoacetate when treated *in vitro* with rat liver homogenates or with washed liver particulate matter. The similarity of the oxidative mechanism of sorbic and caproic acids is further indicated by the fact that they both required orthophosphate for the maximum rate of formation of acetoacetate when the fatty acids were oxidized *in vitro* with washed particles of rat liver as the source

[a]Carried out under a research grant from The Best Foods, Inc.

[b]Paper No. 347 from the Department of Biochemistry and Nutrition, University of Southern California.

[c]The material presented in this report comprises part of two papers (No. 41 and 82) presented at the Boston meeting of the Institute of Food Technologists on June 22-24, 1953.

of fatty acid oxidase [Witter, Newcomb, and Stotz (17)]. In fact, in the most recent report from this same laboratory *(18)*, in which the role of adenosinetriphosphate in the activation of fatty acid oxidation *in vitro* is discussed, it is stated that "sorbic acid, an unsaturated 6-carbon acid, was used as a model substrate."

Although the results of Cohen *(5)* and of Witter *et al. (16-18)* are convincing for the isolated liver system, it remains to be demonstrated whether or not sorbic acid will behave in the same manner when metabolized *in vivo*. It must also be shown that the ketone bodies formed under such conditions will largely disappear from the urine as the result of an oxidation when carbohydrate is fed simultaneously with them.

In order to determine whether or not sorbic acid follows the same pathway of oxidation *in vivo* as the natural 6-carbon fatty acid, caproic acid, experiments were carried out on rats. No information would be obtained by feeding these acids to rats having access to food, since caproic acid and other even-number carbon acids are known to be oxidized completely to carbon dioxide and water under these conditions. The desired answer, however, can be obtained when the fatty acids are fed to *fasting* rats. In this situation, the rats will excrete largely the intermediate ketone bodies. Rats develop a ketonuria of small magnitude when fasted, and the ketonuria is not sufficiently great for comparative studies *(4, 6, 14)*. However, when the fasting rats are given ketogenic acids, they develop a ketonuria of considerable magnitude which is referred to as an *exogenous ketonuria*.

The procedure for developing exogenous ketonuria in the rat provides an excellent method for comparing, both qualitatively and quantitatively, the intermediary metabolism of the several fatty acids. The results of Butts *et al. (3)* and of Deuel and co-workers *(10)* demonstrated that the feeding of equimolecular amounts of butyric, acetoacetic, and caproic acid (as their sodium salts) to rats results in the production of identical levels of ketonuria while the administration of a similar dosage of caprylic (octanoic) acid results in the elimination of about double the amount of ketone bodies *(3, 10)* as results from the aforementioned acids. Higher even-chain fatty acids when given in equimolecular amounts produce a still higher degree of ketonuria than obtains with caprylic acid *(10)*. On the other hand, the administration of odd-chain acids results in practically no ketonuria *(10)*.

The procedure of exogenous ketonuria can also be used to obtain valuable confirmatory information on sorbic acid homologues. Thus, a comparison of the ketonuria following the feeding of equimolecular amounts of crotonic acid, $CH_3 \cdot CH : CH \cdot COOH$, and of butyric acid as their sodium salts would establish whether or not the sorbic acid-caproic acid relationship is a general one between β-unsaturated fatty acids and the corresponding saturated acids.

Studies were therefore made in one series of tests on the ketonuria resulting when equimolecular doses of sorbic, caproic, and butyric acids were given as their sodium salts to fasting rats without or with the simultaneous administration of glucose. In a second series, a comparison was made of the extent of the ketonuria following the administration of butyric or crotonic acids as their sodium salts to fasting rats; a comparison of the

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distribution of the ketone bodies in the urine after these acids were given was also investigated.

EXPERIMENTAL

The procedures employed were similar to those used in our earlier tests *(4)*. The animals were fasted for one day before the start of the experiment after which the body weight was determined on the morning of the first day. The animals were fed the fatty acids as their sodium salts by stomach tube at levels of 75 or 150 mg. (calculated as acetone) per 100 sq. cm. of body surface per day in divided doses in the morning and evening. In those cases in which glucose was also given, it was fed at a level of 75 mg. per 100 sq. cm. when the acids were given at the lower level and at 100 mg. per 100 sq. cm. for the second to fourth day at 1,000 mg. per rat on the fifth day in the tests in which the ketogenic acids were fed at the higher level (150 mg.). Body surface was calculated by the formula of Lee *(13)* based on the weight on the first fast day.

The rats were from the University of Southern California colony which had previously received the stock diet. Because of the higher ketonuria in the female rats as compared with the male rats *(4)*, only female animals were used in these tests. The dosages of ketogenic acids chosen were ones which had been shown by previous experience to be sufficiently high to provide a marked ketonuria but ones which were ordinarily not toxic.

During the urine collection the rats were kept in circular cages which were supported on glass funnels equipped with flasks below for collecting the samples. Cones of wire gauze were used in the funnels to trap the feces and so prevent contamination of the urine. Mineral oil was added to the collecting flasks so that it would cover the urine and so prevent the loss of acetone. The urine samples were collected once daily in the morning. The funnels were rinsed and the sample in the flask together with the washings were made up to volume. The urine from the first day was discarded as this was considered to have little importance during the orientation of the animals to the fasting regimen.

Animals were supplied drinking water *ad libitum* during the course of the tests. Solutions of the sodium salts of the fatty acids and of glucose when given were administered in the morning (after the collection of the urine sample) and at 5 P.M. The experiments were usually continued for 5 days, although the crotonate tests were continued for only 4 days.

Approximately 20% aqueous solutions of the sodium salts of fatty acids were used in the feeding tests. The solutions were prepared by dissolving weighed amounts of the fatty acids in water with the addition of concentrated sodium hydroxide solution until the pH was adjusted to 7.40. The solutions were kept in the refrigerator between feedings. They were found to remain stable for at least a week under these conditions.

Aliquots of the urine samples were analyzed for total acetone bodies and for the separate acetone body fractions by the gravimetric procedure of Van Slyke *(15)*. The identity and purity of the samples of fatty acids were determined in each series of tests either in our own laboratories or in those of The Best Foods, Inc. as described in the earlier paper *(7)*. Sorbic acid was obtained from the Union Carbide and Carbon Company, New York; butyric acid and caproic acid were purchased from the Matheson Co., East Rutherford, New Jersey; and Eastman crotonic acid was used. The latter acid was purified by recrystallizing it twice. In this case, the purity of the product was established by melting point and equivalent weight determinations.

RESULTS

Tables 1 and 2 record the ketonuria which obtains in fasting rats receiving sorbic, caproic, or butyric acids as sodium salts at 75 and 150 mg. per 100 sq. cm., respectively, alone or with glucose. The data on the ketonuria tests following the administration of sodium crotonate and sodium butyrate are included in Table 3.

The data presented in Tables 1 and 2 indicate that the path of metabolism of sorbic acid is identical with that of the corresponding 6-carbon saturated acid, caproic acid, and with that of the 4-carbon derivative, butyric acid. This conclusion is based on the fact that, when corresponding doses of the sodium salts of these acids were fed to fasting female rats, the proportion excreted in the urine as ketone bodies was approxi-

TABLE 1

The ketone body excretion in the urine of fasting female rats when fed butyric, caproic, or sorbic acids as their sodium salts in doses of 75 mg. daily (calculated as acetone) per 100 square centimeters surface area, without or with glucose

Category	Tests with fasted rats			Tests with glucose-fed rats		
	Butyrate	Caproate	Sorbate	Butyrate	Caproate	Sorbate
Ketogenic acid fed:						
Calculated as acetone, mg./100 sq. cm.	75	75	75	75	75	75
Calculated as acid, mg./100 sq. cm.	115	150	145	115	150	145
Average total daily dose fed, mg.	372	482	458	369	474	463
Glucose fed, mg./100 sq. cm.	0	0	0	75	75	75
Urinary ketone bodies, mg./100 sq. cm.[a]						
2nd day	13.3(16)	17.8(16)	12.1(15)	3.6(6)	8.5(6)	3.3(7)
3rd day	18.0(16)	21.9(15)	18.8(16)	5.6(6)	11.6(6)	5.0(7)
4th day	20.2(15)	26.3(13)	25.0(15)	6.7(6)	14.3(6)	9.8(5)
5th day	27.4(13)	29.9(15)	23.8(13)	6.2(6)	16.2(6)	6.8(4)
Average 4-5 days[b]	23.8±2.1 (28)	28.1±2.1 (28)	24.4±1.4 (28)	6.4±0.9 (12)	15.2±2.0 (12)	8.3±1.7 (9)
Reduction from control, mg.	....	....	....	17.4	12.9	16.1

[a] Figures in parentheses indicate the number of experiments included in the average.

[b] Including the Standard Error of Mean calculated from the formula $\sqrt{\Sigma d^2/n-1}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

mately the same. The results were identical, irrespective of whether the acids were administered at a level of 75 mg. or 150 mg. daily (calculated as acetone) per 100 sq. cm. of body surface. When the acids were fed at the lower dosage, 37 and 33%, respectively, of the amount of caproic and sorbic acid appeared as acetone bodies in the urine. When the acids were fed at the higher level, 47 and 41%, respectively, of the caproic and sorbic acid fed were excreted as ketone bodies in the urine. These results are considered to be identical within experimental error. They indicate that the normal pathway of oxidation of both caproic and sorbic acids is *via* ketone bodies.

In order to demonstrate that this ketonuria following the ingestion of sorbic acid is physiological, data are recorded on the effect of moderately sized doses of glucose in suppressing it. In the tests in which the ketogenic acids were fed at the lower level (75 mg.), as recorded in Table 1, the ketonuria was reduced by the 75 mg. dose of glucose somewhat more in the case of sorbate than with caproate, but to the same extent as when butyrate was given. When the ketogenic acids were given at the level of 150 mg. (Table 2), there was also a marked reduction in the ketonuria from the second to the fourth days when the glucose was given at the level of 100 g. per sq. cm. A practically complete disappearance of ketonuria was obtained on the fifth day when 1,000 mg. of glucose was fed. These results indicate that the ketonuria observed after sorbate is identical with that produced after the administration of the normally-occurring fatty acids. Under these conditions of normal alimentation, it is evident that sorbic acid is completely oxidized to carbon dioxide and water, as are the fatty acids normally present in fats; sorbic acid thereby would yield its entire 6.63 Cal. per g. for energy.

The data presented in Table 3 indicate that crotonic acid and butyric acid also have an identical intermediary metabolism. Not only were similar amounts of acetone bodies produced when equimolecular quantities of the sodium salts of these acids were given to fasting rats, but also the proportion of the total ketone bodies comprised by acetoacetic acid is the same within experimental limits. Blunden *(1)* concluded that "In all probability, crotonic acid is an intermediate in the direct oxidation of butyric acid."

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or with glucose

Tests with glucose-fed rats		
utyrate	Caproate	Sorbate
75	75	75
115	150	145
369	474	463
75	75	75
3.6(6)	8.5(6)	3.3(7)
5.6(6)	11.6(6)	5.0(7)
6.7(6)	14.3(6)	9.8(5)
6.2(6)	16.2(6)	6.8(4)
6.4±0.9	15.2±2.0	8.3±1.7
(12)	(12)	(9)
17.4	12.9	16.1

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TABLE 2

The ketone body excretion in the urine of fasting female rats when fed butyric, caproic, or sorbic acids as their sodium salts in doses of 150 mg. daily (calculated as acetone) per 100 square centimeters surface area, without or with glucose

Category	Tests with fasted rats			Tests with glucose-fed rats		
	Butyrate	Caproate	Sorbate	Butyrate	Caproate	Sorbate
Ketogenic acid fed:						
Calculated as acetone, mg./100 sq. cm.	150	150	150	150	150	150
Calculated as acid, mg./100 sq. cm.	230	300	290	230	300	290
Average total daily dose fed, mg.	757	990	983	790	1030	990
Glucose fed:						
2nd-4th days, mg./100 sq. cm.	0	0	0	100	100	100
5th day, mg.	0	0	0	1000	1000	1000
Urinary acetone bodies, mg./100 sq. cm.[1]						
2nd day	25.1(18)	34.5(20)	34.9(16)	6.2(10)	14.8(10)	9.4(9)
3rd day	43.2(16)	55.1(19)	39.9(17)	8.4(10)	16.4(10)	20.0(8)
4th day	64.9(16)	67.9(19)	58.6(13)	10.8(9)	22.2(10)	27.8(8)
5th day[2]	70.2±3.6 (14)	74.5±4.1 (16)	63.0±3.3 (9)	1.3±0.2 (9)	4.5±0.2 (9)	4.9±0.3 (7)
Average:[2]						
4-5 days	67.5±4.1 (30)	71.2±3.3 (35)	60.8±3.5 (22)	....	....	....
2-4 days	....	....	....	8.4±0.7 (29)	17.8±1.2 (30)	18.7±2.4 (25)
Reduction from control, mg.[3]	....	....	....	68.9	70.0	58.1

[1] Figures in parentheses indicate the number of experiments included in the average.
[2] Including the Standard Error of Mean calculated as in Table 1.
[3] Calculated for 5th day only.

TABLE 3

The ketone body excretion in the urine of fasting female rats when fed butyric or crotonic acids as sodium salts in doses of 150 mg. (calculated as acetone) per 100 square centimeters surface area[1]

Category	Series I		Series II	
	Butyrate	Crotonate	Butyrate	Crotonate
Average body weight, gm.	135	131	136	132
Urinary ketone bodies, mg./100 sq. cm.[2]				
1st day	38.0(4)	28.4(6)	72.3(4)	66.7(4)
2nd day	45.5(4)	42.5(6)	84.2(3)	78.8(3)
3rd day	61.8(4)	47.6(6)	77.5(3)	48.7(3)
Average	48.4(12)	39.6(18)	77.4(10)	64.9(10)
Acetoacetic acid, %				
1st day	28.6	32.7	27.1	27.8
2nd day	30.0	31.4	24.9	28.2
3rd day	27.6	32.3	26.0	24.4
Average	28.8	32.1	26.0	26.8

[1] Data from dissertation of Blunden *(1)*.
[2] Figures in parentheses indicate the number of experiments included in the average.

DISCUSSION

The present results establish the fact that the pathway of metabolism of sorbic acid and of its 4-carbon homologue, crotonic acid, is by way of the ketone bodies in the case of rats. The conclusions can now be based on quantitative data obtained *in vivo* which are supported by earlier *in vitro* tests made on liver slices or homogenates.

The question naturally arises as to whether or not the results obtained by the procedure of exogenous ketonuria on the rat employed here has general application. There are several facts which relate the identity of ketonuria in man with that in the rat. In the first place, an exogenous source of fatty acids will increase the degree of ketonuria in both species. Secondly, the ketonuria in both the human and rat is abolished by concomitant ingestion of metabolizable carbohydrate. Thirdly, the markedly higher level of ketonuria occurring in women during fasting as contrasted with that occurring in men under the same conditions *(8)* is exhibited also in exogenous ketonuria both in rats and in guinea pigs *(4)*. The same sex difference in ketonuria occurs as well in another type of experimental ketonuria in rats, namely in *endogenous ketonuria (11, 12)*. And lastly, the greater ketolytic action of galactose over glucose, which was first demonstrated in man *(9)* has been shown to occur in rats when the experimental procedure of exogenous ketonuria was used *(2)*. Thus, it is evident that the identity of the metabolism of sorbic acid and caproic acid, as demonstrated by the experiments on rats, must also be true for the human.

SUMMARY

1. The intermediary metabolism of sorbic acid has been shown to be identical with that of the normally-occurring fatty acids; caproic and butyric. The proof of this behavior is based on the demonstration by *in vivo* experiments on rats that an identical ketonuria obtains during fasting when these several acids are administered in equimolecular amounts.

2. When glucose was administered with sodium sorbate to fasting rats, the decrease in ketonuria was shown to be similar to that noted when carbohydrate was given to rats receiving sodium caproate or sodium butyrate.

3. Experiments are recorded in which it was shown that the ketonurias produced by the 4-carbon homologue of sorbic acid, namely crotonic acid, and by butyric acid, are identical both qualitatively and quantitatively.

4. These facts indicate that the α,β-unsaturated fatty acids are intermediates in the oxidation of the corresponding saturated acids.

5. The conclusion is justified that under normal conditions of alimentation, sorbic acid is completely oxidized to CO_2 and H_2O and thus yields its potential energy as calories.

LITERATURE CITED

1. Blunden, H. D. The Intermediary Metabolism of Butyric Acid as Determined by the Physiological Behavior of the Various Four-Carbon Acids. (Dissertation, University of Southern California, 1938.)
2. Butts, J. S. Studies on ketosis. IV. The comparative ketolytic effect of galactose, glucose, and lactose in rats. *J. Biol. Chem.*, 105, 87 (1934).
3. Butts, J. S., Cutler, C. H., Hallman, L. F., and Deuel, H. J., Jr. Studies on ketosis. VI. Quantitative studies on β-oxidation. *J. Biol. Chem.*, 109, 597 (1935).

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J. . Chem., 109, 597

4. Butts, J. S., and Deuel, H. J., Jr. The sexual variation in carbohydrate metabolism. II. The metabolism of diacetic acid in fasting rats and guinea pigs. *J. Biol. Chem.*, 100, 415 (1933).
5. Cohen, P. P. Studies in ketogenesis. *J. Biol. Chem.*, 119, 333 (1937).
6. Cori, G. T., and Cori, C. F. The fate of sugar in the animal body. V. A seasonal occurrence of ketonuria in fasting rats accompanied by changes in carbohydrate metabolism. *J. Biol. Chem.*, 72, 615 (1927).
7. Deuel, H. J. Jr., Alfin-Slater, R., Weil, C. S., and Smyth, H. F. Jr. Sorbic acid as a fungistatic agent for foods. I. Harmlessness of sorbic acid as a dietary component. *Food Research*, 19 (1), 1 (1954).
8. Deuel, H. J., Jr., and Gulick, M. Studies on ketosis. I. The sexual variation in starvation ketosis. *J. Biol. Chem.*, 96, 25 (1932).
9. Deuel, H. J., Jr., Gulick, M., and Butts, J. S. Studies on ketosis. II. The comparative ketolytic action of glucose, galactose, fructose, and sucrose. *J. Biol. Chem.*, 98, 333 (1932).
10. Deuel, H. J. Jr., Hallman, L. F., Butts, J. S., and Murray, S. Studies on ketosis. VIII. Quantitative studies on the oxidation of the ethyl esters of the fatty acids. *J. Biol. Chem.*, 116, 621 (1936).
11. Deuel, H. J. Jr., Hallman, L. F., and Murray, S. Studies on ketosis. XI. The relation of fatty livers to fasting ketonuria in the rat. *J. Biol. Chem.*, 119, 257 (1937).
12. Deuel, H. J., Jr., Hallman, L. F., and Murray, S. Studies on ketosis. XIV. Ketolysis *versus* antiketogenesis as an explanation for the action of carbohydrate on ketonuria. *J. Biol. Chem.*, 124, 385 (1938).
13. Lee, M. D. Determination of the surface area of the white rat with its application to the expression of metabolic results. *Am. J. Physiol.*, 89, 24 (1929).
14. Levine, H., and Smith, A. H. Studies on ketosis in the rat. *J. Biol. Chem.*, 75, 1 (1927).
15. Van Slyke, D. D. Studies on acidosis. VII. The determination of β-hydroxybutyric acid, acetoacetic acid, and acetone in urine. *J. Biol. Chem.*, 32, 455 (1917).
16. Witter, R. F., Newcomb, E. H., and Stotz, E. The oxidation of hexanoic acid and derivatives by liver tissue *in vitro*. *J. Biol. Chem.*, 185, 537 (1950).
17. Witter, R. F., Newcomb, E. H., and Stotz, E. Influence of orthophosphate on the oxidation of hexanoate and its derivatives *in vitro*. *J. Biol. Chem.*, 195, 663 (1952).
18. Witter, R. F., Newcomb, E. H., and Stotz, E. The role of adenosine triphosphate in the activation of fatty acid oxidation *in vitro*. *J. Biol. Chem.*, 200, 703 (1953).

FURTHER EXPERIENCE WITH THE RANGE FINDING TEST IN THE INDUSTRIAL TOXICOLOGY LABORATORY*

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THREE years ago we presented a paper (1) describing the Range Finding Test, which quickly and economically estimates the magnitude of some of the important health hazards of new chemicals. So far as we are aware and much to our surprise, there has been an absence of criticisms. We must confess we had temerity in setting forth such a simplified procedure in an age of growing refinements in techniques. A philosophy similar to ours has been admitted by Bratton (2), who describes a seven-day feeding test with mice which he employed with satisfaction for the preliminary examination of antimalarial agents. Recently one of us has had occasion to explain in some detail a program for the study of new chemicals, and to show the relation of the range finding test to this activity (3).

Far from weakening our faith, the passage of time has increased the value which we attach to this collection of abbreviated procedures. It is only by the use of short-cuts that our group has been able to accommodate in its schedule a reasonable amount of work which we consider advanced, to distinguish it from investigations of the caliber of the range finding test. The latter fills in our repertory a very real need for predicting the order of magnitude of the toxic hazards to be encountered in the production and utilization of a new chemical. The predictions may be sufficiently clear and favorable so that the compound is obviously no more hazardous to produce than a very familiar product; or so unfavorable that it is not manufactured until specially designed equipment can be obtained for synthesizing it safely; or the manufacture may be postponed because the applications which are foreseen for the product would be too hazardous in view of its physiological properties. But always it must be borne in mind that Range Finding data are in no sense final or complete, they must be supplemented by advanced studies before wide application of a chemical can be known to be safe. Our series of papers on the polyethylene glycols (4), not yet finished, represents rather concretely the concept of the many-sided advanced study.

Our earlier paper (1) stressed the speed with which sound approximations can be obtained by the range finding test. A period of three weeks was named as the time required for completion of the work. This estimate implied the existence of a staff ready to function upon receipt of a sample, and supplied at all times with suitable animals. In special situations the period elapsing before useful information is available can be much reduced. Within the past few years reassuring or cautionary statements have been issued as promptly as twenty-four hours after a sample was received, later to be amplified at greater leisure. Experience has now shown that such celerity is seldom required, and that it is uneconomical, if not actually impossible, to maintain a laboratory prepared to commence all the procedures of the range finding test the moment an express shipment is received.

Experience has further revealed that in the majority of cases some of the steps of the test may be omitted without prejudicing the applicability of the results to the immediate need. More and more as time passes, the steps performed are tailored to fit the specific need. A chemical may have such a low vapor pressure that inhalation is obviously of no concern to the industrial hygienist, a sample may be so scant that only those tests requiring minimum volumes can be performed, or the question accompanying the sample may be so specific that only one operation is required to obtain an answer. Happily this latter situation arises more often each year as those who originate requests grow more familiar with the nature of toxicological data and their own needs for information. The all-inclusive question, "What is the toxicity of such and such a compound?", which would require a life time to answer, has almost vanished from our correspondence.

There are few standardized procedures which remain unchanged over several years and the details of the range finding test have never been regarded as more than tentative. It is not sur-

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prising therefore, that if the test were to be reduced to formal specifications today, it would be found to differ in several perhaps important respects from the description given in 1944. The differences will be discussed under the head of each separate step in the collection.

Single Dose Oral Toxicity

During the war our supply of Wistar strain rats was eliminated by the drafting of a commercial breeder. Comparison of several available strains allowed us to select the Sherman as an approximation to the Wistar in resistance to the acute toxicity of some of the more important chemicals which concerned us. Accordingly, the loss of the Wistar strain did not vitiate our files of acute toxicity data.

The LD_{50} is still bracketed by the use of groups of 6 rats given dosages differing in a ratio of 10. But now the approximation is often made closer by interpolating two groups of 10 rats, each of which suffers fractional mortality. Our standard dosage series, when expressed in gm./kg., constitutes the antilogarithms of 1.0, 1.1, 1.2, etc., and for range finding tests alternate dosages in the series are employed; the larger therefore exceeds the smaller by 58 per cent. These additional animals do not materially increase the time required for the test.

It is recognized that dependence upon one species is hazardous, accordingly for advanced studies other species are added.

Sub-acute Oral Toxicity

One of the serious deficiencies of the earlier description was the absence of any criterion of chronic toxicity. This defect has been remedied by administering doses to groups of 10 rats each, 5 of each sex in one cage, for thirty days. The sample is incorporated in a diet of 1000 gms. ground whole wheat, 500 gms. dried whole milk, and 20 grams of iodized salt, freely available to the animals in modified McCollum feeding cups from which very little is spilled. Water is provided in siphon drinking bottles and rarely it, rather than the diet, is the carrier of the chemical. The diet for each cage is kept in a separate jar which is weighed before and, together with uneaten food in the cup, after the thirty-day period. Three or four dosage levels differing by a ratio of 2 or 4 are administered, together with a control diet.

The criteria of injury in this step are effects on the increase in body weight from the approximate 120-gram starting weight, reduction in appetite, death, and micropathology of adrenal, upper intestine, kidney, liver and spleen. Table 1 sets forth typical results from such a test. It is apparent from the data that dosages were selected with the aim of allowing at least one group of rats to survive without injury as judged by the limited but sensitive criteria, rather than causing many fatalities. If the latter goal is pursued many rats will voluntarily starve themselves owing to the objectionable taste of diets containing high levels of chemical, and one will be uncertain of the cause of death. Daily forced feeding would avoid this doubt but would entail too much labor to be justified in a range finding test. Usually pathological study adds little quantitative information to the other observations, and it is doubtful if the operation is worthwhile unless the thirty-day feeding is regarded as a preliminary step to a two-year study where advanced work on a chemical justified the expense.

Planning of dosage levels is simplified by the observation that, under the conditions specified, a level of 1 per cent in the diet results in an average dosage of close to 0.6 gm./kg./day and 1 per cent in the water produces 0.8 gm./kg./day.

Skin Absorption

The factor of absorption through the intact skin grows more important each year. The previous communication (1) noted a shift from the four-day poultice upon guinea pigs to the one-day rubber cuff application upon rabbits of the Food and Drug Administration (5). The change is now complete and poultice data are regarded as obsolete because the saturated pad prevents complete contact of the dose with the skin. Dosages as great as 20 ml./kg. can be retained by the cuff, or dam, as it is known to us. The numbers of animals used are similar to those given oral dosages.

Vapor Exposure

The estimation of hazard by inhalation of saturated vapors has been amplified by adding the inhalation of vapors of known concentration, aiming at a concentration which produces fractional mortality as a result of a four-hour exposure. Concentrations used fall in a series differing by a ratio of 2, and they are approximations because they are estimated from the settings of a proportioning pump and flowmeter rather than being determined analytically. The latter procedure is

Typical R
Materials Administered

Material studied
Acetophenone
Acetylethanolamine
Antimony trioxide
Butyl "Carbitol" (3, 4)
Butyl phthalyl butyl glycolate
Calcium ethyl acetoacetate
"Carbitol" solvent (3, 4)
"Carbitol" acetate (3, 4)
Diacetone alcohol (3)
Dibutyl "Cellosolve" phthalate
Diethylbutyl "Cellosolve" succinate
Diethylene glycol ethyl ether (pure) (3, 4)
Ethoxy triethylene glycol (3)
Ethylhexanimido diethyl di(ethyl hexanoate)
Glycidyl oleate
Heptadecyl glyoxalidine hydrochloride (3)
Methyl Acetyl Ricinoleat
Methyl "Carbitol" (3, 4)
Methyl "Cellosolve" ace Ricinoleate
Methyl phthalyl ethyl glycolate
Propylene glycol ethyl ether (beta isomer) (3)
Sodium benzoate
Sodium sorbate
Sorbic acid
"Tergitol" penetrant 08 (3, 4)
"Tergitol" penetrant 4 (3, 4)
"Tergitol" penetrant 7 (3, 4)
Triisopropanolamine (3,
Triethylhexyl phosphat

(1) Dosage level pro
(2) When LD_{50} was in parentheses. The le that the dosage shown i
(3) Dose administer consumed.
(4) Only 5 rats to a

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TABLE 1

TYPICAL RESULTS WITH RANGE FINDING TEST FOR SUB-ACUTE ORAL TOXICITY

Materials Administered in the Food to Groups of 10 Rats for 30 Days on each Dosage Level Unless Noted Otherwise

MATERIAL STUDIED	RANGE OF DOSAGE LEVELS FED		LEAST DAILY DOSAGE CAUSING SYMPTOM				MAXIMUM DOSAGE HAVING NO EFFECT	SINGLE DOSE ORAL LD_{50} (2)
	Maximum	Minimum	Reduced growth	Reduced appetite	Micropathology (1)	Death		
	gm./kg.	gm./kg.	gm./kg.	gm./kg.	gm./kg.	gm./kg.	gm./kg.	gm./kg.
Acetophenone	0.102	0.001	—	—	—	—	>0.102	0.90 (0.81 to 1.00)
Acetylethanolamine	1.93	0.30	—	—	—	—	>1.93	27.66 (25.24 to 30.31)
Antimony trioxide	1.07	0.06	1.07	1.07	1.07	—	0.27	>20
Butyl "Carbitol" (3, 4)	1.83	0.051	—	0.094	0.65	—	0.051	6.56 (5.47 to 7.86)
Butyl phthalyl butyl glycolate	1.56	0.10	1.56	—	1.56	—	0.45	14.64 (11.73 to 18.28)
Calcium ethyl acetoacetate	0.34	0.014	—	—	0.060	—	0.014	9.93 (9.22 to 10.70)
"Carbitol" solvent (3, 4)	3.20	0.41	1.83	3.20	0.41	—	<0.41	9.05 (8.43 to 9.72)
"Carbitol" acetate (3, 4)	3.20	0.23	3.20	1.80	0.23	3.20	<0.23	11.00 (10.40 to 11.59)
Diacetone alcohol (3)	0.13	0.01	—	—	0.04	—	0.01	4.0 RF
Dibutyl "Cellosolve" phthalate	1.74	0.11	1.74	1.74	1.74	—	0.50	8.38 (6.86 to 10.23)
Diethylbutyl "Cellosolve" succinate	1.44	0.17	1.44	1.44	0.44	0.17	<0.17	3.3 RF
Diethylene glycol ethyl ether (pure) (3, 4)	3.88	0.21	1.77	0.87	1.77	—	0.49	8.69 (7.25 to 10.41)
Ethoxy triethylene glycol (3)	3.30	0.18	3.30	3.30	3.30	3.30	0.75	10.61 (9.98 to 11.28)
Ethylhexanimido diethyl di(ethyl hexanoate)	1.53	0.10	—	—	—	—	>1.53	>39.8
Glycidyl oleate	0.35	0.014	—	—	—	—	>0.35	3.52 (3.35 to 3.69)
Heptadecyl glyoxalidine hydrochloride (3)	0.26	0.06	0.16	—	0.26	0.26	0.06	1.97 (1.70 to 2.32)
Methyl Acetyl Ricinoleate	1.82	0.12	—	—	—	—	>1.82	>50
Methyl "Carbitol" (3, 4)	1.83	0.19	1.44	0.79	0.19	—	<0.19	9.21 (7.90 to 10.71)
Methyl "Cellosolve" acetyl Ricinoleate	1.50	0.11	1.50	1.50	—	—	0.45	20.27 (15.13 to 27.15)
Methyl phthalyl ethyl glycolate	1.90	0.11	—	—	1.90	—	0.48	9.06 (8.11 to 10.13)
Propylene glycol ethyl ether (beta isomer) (3)	2.14	0.16	2.14	2.14	—	—	0.68	8.93 (7.89 to 10.09)
Sodium benzoate	1.09	0.016	—	—	—	—	>1.09	4.07 (3.72 to 4.44)
Sodium sorbate	1.08	0.016	—	—	—	—	>1.08	7.16 (6.82 to 7.52)
Sorbic acid	1.27	0.011	0.048	—	0.048	—	0.011	7.36 (6.69 to 8.90)
"Tergitol" penetrant 08 (3, 4)	1.51	0.23	1.24	1.24	0.80	—	0.44	10.32 (9.05 to 11.45)
"Tergitol" penetrant 4 (3, 4)	0.73	0.22	0.35	0.35	0.22	—	<0.22	4.95 (4.42 to 5.53)
"Tergitol" penetrant 7 (3, 4)	0.74	0.25	0.55	0.36	0.41	—	0.25	5.66 (4.85 to 6.60)
Triisopropanolamine (3, 4)	1.35	0.14	1.35	1.35	0.26	—	0.14	6.50 (5.62 to 7.54)
Triethylhexyl phosphate	1.55	0.11	1.55	—	—	—	0.43	37.08 (33.70 to 40.79)

(1) Dosage level producing micropathology in liver, kidney, spleen or testis of any rat.

(2) When LD_{50} was derived from an advanced test the range of 1.96 times the standard deviation is shown in parentheses. The letters "RF" refer to data derived from a range finding test. The symbol ">" indicates that the dosage shown is the maximum which could be administered and it killed none of 6 rats.

(3) Dose administered in the drinking water. In this case appetite is judged on the basis of ml. of water consumed.

(4) Only 5 rats to a dosage level.

TABLE 2

Typical Results with Range Finding Test

Capital Letters Designate Chemicals Having the Respective Hazard in the Same Order of Magnitude as the Material Studied, and Refer to the List Following the Table

Material Studied	Single Dose Oral Toxicity to Rats		Penetration of Rabbit Skin*		Saturated Vapor Inhalation by Rats		Inhalation of Known Vapor Concentration by Rats				Primary Skin Irritation	Eye Injury from Fluid	
	R.F. LD_{50}		R.F. LD_{50}		Maximum for no death		Concentration	Time	Mortality in 14 days			Grade	
	gm./kg.		ml./kg.				PPM.	hrs.					
Acetol	2.2	A	>16.0	U	—	—	2000	8	0/6	O	V	5	V
Acetonitrile	3.8	E	5.0	O	—	—	8000	4	1/6	Q	V	5	V
Acrylonitrile	0.093	N	(0.25)	N	1 min.	A′	500 1000	4 4	0/6 6/6	L	V	4	U
Allyl alcohol	0.064	N	0.053	A′	—	—	1000	1	4/6	A′	V	5	V
Allyl chloride	0.7	B	2.2	L	—	—	2000	4	1/6	A	V	2	Z
Allylidene diacetate	0.25	A′	0.32	N	—	—	8	4	4/6	T	C′	10	W
Chloropropane diol—1,3	0.11	N	0.45	B	—	—	1000	1	5/6	N	V	4	U
Chlorostyrene	5.2	O	20.0	Z	8 hr.	Z	—	—	—	—	U	2	Z
Cyanomethyl acetate	0.032	>N	0.043	A′	—	—	16 32	4 2	0/6 6/6	T	V	5	V
Diacetone alcohol	4.0	O	14.5	Z	8 hr.	Z	—	—	—	—	V	5	V
Dichloroethyl ether	0.075	N	(0.3)	N	—	—	1000	¾	3/6	A′	U	4	U
Dichloroethyl formal	0.065	N	(0.17)	N	—	—	60 120	4 4	0/6 6/6	N	V	1	Z
2,3-Dichloropropanol	0.09	N	0.2	N	—	—	500	4	2/6	L	U	5	V
Dichlorostyrene	4.0	O	9.0	I	8 hr.	Z	—	—	—	—	T	5	V
Dimethyl dioxane	3.0	O	>10.0	Z	—	—	8000	4	2/6	Q	B′	5	V
Dimethyl formamide	7.0	J	5.0	O	4 hr.	I	—	—	—	—	B′	4	U
Epichlorohydrin	0.09	N	1.3	A	—	—	250	8	4/6	A′	U	4	U
Ethoxy propionaldehyde	0.9	B	1.0	A	—	—	500	4	6/6	A′	V	7	N
Ethoxy propionic acid	4.8	E	0.75	A	—	—	2000	4	0/6	A	C′	9	W
Ethyl chlorobenzene	5.0	O	17.5	Z	8 hr.	Z	—	—	—	—	B′	2	Z
Ethyl dichlorobenzene	5.5	O	14.2	Z	8 hr.	Z	—	—	—	—	C′	1	Z
Ethylene imine	0.015	>N	(0.014)	A′	—	—	50 250	8 2	5/6 3/6	T	T	7	N
Ethylidene acetone	0.32	A′	0.5	B	—	—	250	4	3/6	A′	U	8	B
Hexane diol—2,5	5.0	O	16.3	Z	8 hr.	Z	—	—	—	—	V	3	S
Isopropanol	5.84	V	16.4	Z	—	—	16000	8	4/6	Q	—	4	U
Methyl acetoacetate	3.0	O	(>10)	Z	8 hr.	Z	—	—	—	—	U	7	N
Methyl acrylate	0.3	A′	1.3	A	—	—	1000	4	3/6	L	U	4	U
Methylbenzyl "cellosolve"	2.29	A	5.0	O	4 hr.	I	—	—	—	—	—	3	S
Methyl "cellosolve" acetate	3.39	O	5.25	O	—	—	7000	4	2/6	Q	—	2	Z
Methyl diacetoacetate	1.7	A	(>10)	Z	8 hr.	Z	—	—	—	—	C′	5	V
2-Methyl pentane-2,4-diol	4.70	O	13.30	Z	8 hr.	Z	—	—	—	—	U	4	U
Morpholine ethanol	12.06	S	(2.5)	L	8 hr.	Z	—	—	—	—	V	5	V
Propylene imine	0.019	>N	(0.043)	A′	—	—	500	4	5/6	L	—	9	W
Propylene oxide	1.14	B	(8.64)	I	—	—	4000	4	6/6	A	V	5	V
Tetramethyl ethylene diamine	1.58	A	7.0	I	10 min.	L	—	—	—	—	B′	8	B
Vinyl acetate	2.92	O	2.5	L	—	—	4000	4	3/6	O	V	2	Z

* Figures in parentheses were determined by poultices on guinea pigs.

Comparative Materials Referred to by Letter Code in Table 2

The words "Cellosolve" and "Carbitol" are trade marks of Carbide and Carbon Chemicals Corporation for the solvents that are ethers of ethylene and diethylene glycol respectively. These letter codes have the same significance as in our 1944 article (1). The symbol > before a letter in the table means significantly more toxic than

A	Methyl "Cellosolve" solvent	S	Ethanol
B	Formaldehyde	T	Dimethyl sulfate
E	Lactic acid	U	Ethyl acetoacetate
I	"Cellosolve" acetate solvent	V	Acetone
J	Triisopropanolamine	W	Ammonium hydroxide (28% NH_3)
L	Mesityl oxide	Z	"Carbitol" solvent
N	Ethylene chlorhydrin	A′	Crotonaldehyde
O	"Cellosolve" solvent	B′	Benzene
Q	Cyclohexanone	C′	Diethanolamine

impractical if a new vapor is to be handled every day.

At times there is appraised the hazard of certain operations which result in the creation of a mist by bubbling air through the sample held at the arbitrary temperature of 170° C., cooling the air, and furnishing it to animals in a small chamber. Limited data obtained in this way have been published (6). An attempt is being made to standardize the creation of a fog by the use of a nebulizer, but uniform conditions have not yet been attained. This exposure would reduce the oxidation of samples, which may be important in the 170° mist test.

Skin Irritation

Tests of skin irritation are very difficult to standardize and quantitate, and no further progress in this direction has been achieved.

Eye Injury

A full description of the eye injury test has recently appeared (7) together with a large amount of data, and no time will be spent upon the procedure here.

Skin Sensitization

A second deficiency recognized in the range finding test was the absence of any test for sensitization. During the past two years the Landsteiner guinea pig skin sensitization procedure has been studied. After standardization of an intradermal technique which appeared to yield reliable results, the test was applied to some 40 chemicals of widely different compositions. For at least half of these chemicals there were available for comparison the results of human patch tests, human experience, or experience with closely similar molecules. The results were disappointing. It was found that the guinea pig test did not miss chemicals which were active human sensitizers but that it did condemn others which have been used for years upon human skins without objectionable reactions. It was found to cost as much to perform as a human patch test series done by a consulting dermatologist and to require as much time. The guinea pig test therefore offers no advantages over human application and is no longer regarded as a part of the range finding test.

Materials for which preliminary skin sensitization data are desired are assembled in groups of 8 and referred to a consulting dermatologist who applies them to 30 human subjects. The schedule is one week of contact, about two weeks for development of sensitivity, and two to three days second contact to detect sensitization.

Discussion

Table 2 presents examples of the data obtained with the range finding test. Skin sensitization has not been included and sub-acute oral data are in Table 1. It will be seen that we still lean heavily upon comparisons with more familiar molecules. This part of the test is perhaps the most indefensible because of pharmacological differences between even close analogs. But it is so valuable in allowing industrial technologists to visualize the meaning of toxicological information in terms of chemicals they hand'e every day, that the comparison is indispensable.

It must be emphasized that the range finding test is not believed to yield final or complete toxicological data. Its results are sound indications, but they remain tentative until confirmed and extended. The test can condemn a particular

application of a chemical but it cannot establish its safety. It must also be emphasized that the components are separable, and that rarely are all the steps performed upon a single material. The parts selected depend upon the information desired.

Summary

There are presented elaborations of the Range Finding Toxicity Test in respect to sub-acute toxicity, vapor inhalation, and skin sensitization. Further examples of the use of the test are given.

REFERENCES

(1) Smyth, H. F., Jr. and Carpenter, C. P.: The place of the range finding test in the industrial toxicology laboratory. This J., *26:* 269, 1944.

(2) Bratton, A. C., Jr.: A short-term chronic toxicity test employing mice. J. Pharmacol. & Exper. Therap., *85:* 111, 1945.

(3) Smyth, H. F., Jr.: Solving the problem of the toxicity of new chemicals in industry. W. Va. Med. J., *42:* 177, 1946.

(4) Shaffer, C. B. and Critchfield, F. H.: Solid polyethylene glycols, quantitative determination in biological materials. Analytical Chemistry, *19:* 32, 1947.

Shaffer, C. B. and Critchfield, F. H.: Absorption and excretion of "carbowax" compounds. J. Am. Pharm. Assoc., *36:* 152, 1947.

Smyth, H. F., Jr., Carpenter, C. P. and Shaffer, C. B.: The toxicity of high molecular weight polyethylene glycols; chronic oral and parenteral administration. J. Am. Pharm. Assoc., *36:* 157, 1947.

(5) Draize, J. H., Woodard, G. and Calvery, H. O.: Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. J. Pharmacol. & Exper. Therap., *82:* 377, 1944.

(6) Shaffer, C. B., Carpenter, C. P. and Smyth, H. F., Jr.: Acute and subacute toxicity of di(2-ethylhexyl) phthalate with note upon its metabolism. This J., *27:* 130, 1945.

(7) Carpenter, C. P. and Smyth, H. F., Jr.: Chemical burns of the rabbit cornea. Am. J. Ophth., *29:* 1363, 1946.

UDK (Universal Decimal Classification): 613.2 :

664.8.035 + 664.8.035 : 613.2

Experimental Materials Relative to the Hygienic Characteristics of the Combined Action of Some Chemical Food Preservatives

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The problems involved in our experimental work were the following: to carry out a more profound study and to compare the toxicity of sorbic acid in the Czecholovakian and domestic (Soviet) (laboratory and industrial) industry, and also, of the admixture of a polymer to the latter preparation; to verify foreign literature date relative to the absence of toxicity on the part of nisin in the English industry (at the present time, in the Soviet Union, there are being carried out semi-industrial tests of nisin, and a study is being made of the possibility of obtaining a domestic preparation); to make a more profound investigation of the degree to which toxic properties are pronounced in the preservatives, being utilized, at the present time, by the domestic (Soviet) industry -- sodium bisulfite and benzoic acid; to make a study of the combined effect on the organism of animals of sorbic acid with nisin and of sodium bisulfite with benzoic acid.

The work was carried out on pure preparations of the preservatives. Doses of the preparations were calculated, proceeding from the permissible concentrations in food products, with a safety factor (SF) equal to 1 and 2. Thus, sorbic and benzoic acid were introduced in an amount of 40 and 80 milligrams, sodium bisulfite -- 80 and 160 milligrams, and nisin -- 2 and 4 milligrams to 1 kilogram of animal body weight. In addition to this, the nisin was studied in doses of 0.4, 40, and 400 milligrams/kilogram, and the polymer -- in doses of 0.4 and 800 milligrams/kilogram. Acute experiments were not set up, and subacute ones were conducted in a limited volume. Principal attention was de-

voted to chronic experiments.

In the study, use was made of 1900 white mice and 400 white rats of both sexes and of varying weight (in conformity with the data in the literature, in studying food preservatives, other authors did not set up experiments on mice).

In chronic experiments, the preparations were administered to the experimental animals in accordance with the method of group inoculation. For this, we made ready inoculation mixtures, which consisted of the preservatives and products under study, which were introduced into the diet of the animals. The control animals received the same mixtures but without preservatives. The mixtures were given separately from the general feed.

The statistical treatment of the data obtained was carried out in accordance with the statistics of variations. A determination was made of the arithmetic means, of the quadratic deviations from the means, of the mean errors in the indices, of the errors in the difference of the indices, of the indices of vitality (T, P, chi^2), of the coefficients of correlation and regression depending on the feasibility of making use of the corresponding statistical procedures

There were carried out 3 series of investigations. The duration of the chronic experiments was 8 - 18 months, and that of the subacute one -- 3 months.

In investigating the animals, use was made of more than 30 indices: observation of the overall state, reproduction of progeny, the survival rate under customary experimental conditions and in the case of starvation, the consumption of water and feed, gain in body weight, tests on cooling the organism, physical burdens, tests with carbon tetrachloride and K_2HPO_4, determination of the blood formula, of the complement titer, of the phagocytic activity of the leucocytes and of the exidase activity of the blood, of the ESR (erythrocyte sedimentation rate), according to Efman, of the alkalinity of the blood and of the acetone bodies in the blood, and of the albumin in the urine. In addition to this, there were carried out histopathological investigations, and also, observations of the disclosure of possible carcinogenic and cocarcin-

ogenic action of the preservatives under study.

The animals were divided into the following groups: K -- control; A -- those receiving a mixture of sorbic acid and nisin; ((Russian)) B -- a mixture of sodium bisulfite and benzoic acid; ((Russian)) V -- nisin; ((Russian)) G-- sorbic acid; ((Russian)) D -- sodium bisulfite, and E -- benzoic acid.

The investigations that had been carried out indicated that the highest absolute and relative gain in body weight, and also, the consumption of water and feed, with a simultaneous reduction in the survival rate, both under the customary experimental conditions, as well as in the case of starvation, were noted in the males, both in the mice, as well as in the rats, that had received nisin (group V). Sorbic acid, according to the majority of indices made use of, was disclosed as an antagonist of nisin. Thus, in group A, the body weight, the survival rate, the consumption of water and feed, were within the same limits as in those of the control animals, and in group G, the survival rate in the mice was even 8% higher than in the control. In the animals, particularly in the mice, that had received sodium bisulfite and benzoic acid (separately and in mixture), the indicated indices were lower than in the control ones. Benzoic acid indicated a negative effect on the body weight and on the survival rate of the mice (this did not pertain to the rats). The results are set forth in Tables 1 - 4.

((Table on pg. 62 of text))

From the data set forth in Table 4, it may be seen that the lowest survival rate of male rats, in the case of starvation, is observed in group (R(Russian)) V. In comparison with group ((Russian)) G (there were no males in the control one), this difference is statistically reliable and may be explained by the effect of the preservative, in view of the fact that chi^2, for the initial weight and for the life span of the rats, in the case of starvation, is greater than 99%.

Table 1

Gain in Weight in Mice in 8 Months of the Experiment

(Safety Factor-1)

Group and Sex of the Mice	Initial Weight, 8 - 15 grams			Initial Weight, 16 - 25 grams		
	absolute gain		relative gain (in %)	absolute gain		relative gain (in %)
	in grams	in %		in grams	in %	
K (males..	26.0	185.0	94.40	10.74	53.50	42.47
(females.	14.16	97.6	68.13	15.20	90.03	68.44
A (males ..	28.0	257.0	108.60	18.31	94.03	66.37
(females.	19.3	160.0	93.30	13.90	72.02	55.29
((Russian)) B (males ..	26.67	179.0	105.12	15.55	77.60	67.00
(females.	14.01	108.0	73.67	13.39	72.10	56.14

Table 2

Gain in Weight of the Rats in 17 Months of the Experiment

(Safety Factor-1)

Group and Sex of the Rats	Initial Weight (in grams)	Absolute Gain in grams	Absolute Gain in %	Relative Gain (in %)
K (males ...	111.88	227.65	203.49	111.04
(females ..	101.2	170.8	168.77	98.97
A (males	101.79	258.21	253.67	126.39
(females...	97.54	198.0	202.99	110.85
((Russian)) B (males.....	116.16	191.48	164.84	97.41
(females..	98.40	167.06	151.80	99.42

Table 3

Body Weight, Consumption of Feed (Briquet) and Water, Survival Rate During the Experimental Period and Survival Rate During the Starvation of the Mice (Safety Factor-2)

Group and Sex of the Mice	Absolute Gain in grams	Absolute Gain in %	Relative Gain (in %)	Consumption Per Day of feed (in grams)	Consumption Per Day of water (in milliters	Survival Rate during the experimental period	Survival Rate in the case of starvation (in days)
K (males..	16.57	199.39	109.7	2.33	3.67	60	3.86
(females..	12.13	166.83	93.26	2.81	4.14	72	5.22
A (males....	12.19	128.72	82.74	2.34	3.32	48	10.0
(females..	10.68	114.59	78.35	2.48	4.42	74	5.71
((Russian)) B (males	16.94	174.3	100.9	3.24	3.54	30	3.67
(females	12.24	140.69	87.85	2.32	2.63	38	4.67
((Russian)) G (males....	13.35	145.44	89.78	2.17	3.87	92	4.28
(females..	13.47	144.47	89.36	2.13	3.31	52	4.72
((Russian V (males....	19.20	226.85	118.45	3.36	5.92	12	3.52
(females..	12.47	143.33	87.99	4.31	4.34	32	4.57
((Russian)) D(males.....	13.30	143.01	88.80	2.43	3.38	70	4.71
(females..	11.21	124.14	80.71	1.98	3.13	68	5.64
E (males...	11.28	124.52	80.87	2.57	3.85	68	4.4
(females.	10.95	118.23	78.05	2.65	3.52	68	4.4

((text continued)): Experiments with physical burdens were carried out in the form of swimming (floating) in accordance with the standard procedure, and with an additional load made fast to the tail of the mice, in dimensions of 5 and 50% relative to the body weight. The results are set forth in Table 5.

From Table 5 it may be seen that the greatest endurance with respect to physical burdens was noted in female mice belonging to groups A and ((Russian)) G (with a 5% additional load relative to the body weight). In comparison with groups K, ((Russian)) B, D, this difference is statistically reliable and comprises, respectively, P is less than 0.001, is less than 0.05, and is less than 0.05. At the same time, there has not been established any fixed dependence between the body weight of the mice and their endurance with respect to physical burdens; this makes it possible to relate the difference in the duration of maintaining themselves on the surface of the water to the action of the preservatives under study on the organism.

((Table on pg. 64 of text))

Table 4

Results of Experiment Involving the Starvation of Rats

(Safety Factor-2)

Group and Sex of the rats	Initial Body Weight (in grams)	Loss in Weight (in %)	Survival Rate (in days)	Reliability (P)
K females ...	179.5	36.21	9.75	>0.05
((Russian)) G (males...	225.2	43.7	12.2	
(females..	170.5	38.65	9.5	
((Russian)) D (males...	221.2	43.4	11.8	>0.1
(females..	162.4	39.2	8.8	>0.5
E (males....	193.6	41.0	12.2	
(females..	176.8	36.2	12.0	
((Russian)) V (males....	185.5	39.1	9.25	>0.05
(females..	173.0	37.6	9.5	

((text contihued)): In 8 of the 100 mice in Group B there were disclosed spontaneous tumors, and in the third generation, in one out of 8 mice, whereas in other groups, this was not observed. In connection with the data obtained, in-

vestigations were carried out with respect to the disclosure of a possible carcinogenic and cocarcinogenic action on the part of the preservatives under study and also, with regard to the effect of an excess body weight of the animals on the rate of development of the ascitic Ehrlich cancer cells that had been administered intraperitoneally. The results of these investigations will be published separately. According to the data obtained, benzoic acid and sodium bisulfite, both by themselves, as well as in mixture, possess, to a certain extent, a cocarcinogenic action.

For the purpose of determining the overall detoxication capacity of the liver, we made use of a load with carbon tetrachloride: for the mice, 0.1 milliliter and for the rats, 0.6 milliliter. A single oral administration of it to the animals indicated that sodium bisulfite, benzoic acid and nisin lowered, and sorbic acid and the polymer increased, the detoxication capacity of the liver. The data are statistically reliable.

In the capacity of an overall index for the immunological state of the experimental animals, we made use of a determination of a complement titer and of the phagocytic activity of the leucocytes; for the characteristic of the level for the oxidizing processes, and also, for the functional state of the liver -- of a determination of the oxidase activity of the blood.

The determination of the complement titer was carried out according to the classical method with diminished proportions of the reagents by 10 times and with a dilution of the blood serum by 20 times. The phagocytic activity of the leucocytes was likewise determined in accordance with the classical procedure with the utilization of a 2-milliard (billion) suspension of staphylococcus strain No. 207. The oxidase activity of the blood was determined by means of paraphenylenediamine, in accordance with the Ravin method (1). Examinations of the animals, by these tests, were carried out in the dynamic state, with 10 - 20 experimental rats from each group.

However in connection with the absence of statistically reliable shifts, with the purpose of studying the effect of preservatives on the animal organism

against a background of stress conditions, ae the end of the chronic experiment, all the animals were additionally subdivided into subgroups and were subjected to the influence of cold (in the wintertime) and to overloading in a centrifugal drum; there was also brought about an impairment in the function of the kidneys by means of a load of K_2HPO_4. The results of these investigations are set forth in Table 6.

From the data set forth in Table 6 it may be seen that the highest indices are in troups A and G, under general experimental conditions. In comparison with group B, this distinction is statistically reliable, particularly for the complement titer (P is less than 0.001). In group D there is a very low phagocytic activity on the part of the leucocytes, and of the oxidase activity of the blood. In group E the indices are within the control limits.

((Table on pg. 65 of text))

Table 5

Endurance with Respect to Physical Burdens in Mice

(Safety Factor-1)

Group and Sex of Mice	Swimming Time (in minutes)	
	additional load to the body weight	
	5%	50%
K(males	4.66	5.0
(females.....	5.17	5.5
A (males.....	5.78	27.25
(females.....	26.1	6.52
B (males.....	11.9	16.33
(females.....	13.71	13.55
G (males.....	11.67	--
(females.....	24.17	--
D (males.....	2.6	7.0
(females.....	7.54	5.42
E (males.....	11.71	--
(females.....	16.33	--

((text continued)): Cold and staying in the centrifuge had a more negative effect on group B (in comparison with Group A); at the same time, there was a reduction in all theindices. An experiment on the influence of cold, which had been carried out 8 months prior to this, yielded identical results: in group A, the oxidase activity hardly changed (indications of the optical density were lowered by 0.030), and in group B it was reduced by 0.282. In a subacute experiment (with a Safety Factor-20), the oxidase activity of the blood increased in group A to a larger extent than in group B (indications of the optical density, in the beginning,and at the end of the experiment: in group A -- 0.196 and 0.442; in group B -- 0.161 and 0.623. These data point out that the preservative mixtures under study exert an influence on the oxidizing processes in the organism of the animals: If sorbic acid, in mixture with nisin, under all circumstances, increases the oxidase activity of the blood within biologically favorable limits, then sodium bisulfite (both in mixture with benzoic acid, as well as separately), in small doses, reduces, and large ones, increases, it; this, in both cases, represents a biologically unfavorable property of the given preservative.

Changes, observed in the oxidase activity of the blood, in a chronic experiment, made it possible to assume the presence of possible shifts in the level of acetone bodies in the blood. In determining the acetone bodies in rats, by the Natel'son (2) method, in group K there was disclosed 2.5 milligrams %, in group A -- 3.4 milligrams %, and in group B -- 1.97 milligrams %. However, after 2 weeks following this, as these rats were subjected to a subacute experiment (with a Safety Factor-20), and when the animals were examined for the acetone content in the blood, the following results were obtained: in group K -- 1.53 milligrams %, in group A -- 2.15 milligrams %, and in group B -- 2.88 milligrams %. This points out that an increase in the doses of sorbic acid and nisin was not instrumental in increasing the level of acetone bodies in the blood, whereas large doses of sodium bisulfite and benzoic acid did increase this level.

The data obtained confirm our opinion with respect to the faxt that a mixture of sodium bisulfite and benzoic acid exerts an unfavorable influence on the oxidizing process in the organism of the animals. The distinctions are statistically reliable (P within the range of 0.05).

With a load of K_2HPO_4 (up to 1000 milligrams for a rat per day), for establishing a kidney insufficiency, it was determined that preparations of inorganic phosphorus are of low toxicity and of little use for these purposes. However, the albumin in the urine of the rats in group B was nevertheless somewhat greater (3.3 0/00) than in group A (2.21 0/00); P is greater than 0.1. ((Table on pg. 66 of text))

Table 6

Complement Titer, Phagocytic Activity of the Leucocytes and Oxidase Activity of the Blood after 17 Months From the Beginning of the Experiment in Rats Kept Under Customary Vivarium Conditions and Subjected to the Influence of Stress Factors

Group of Rats	Experimental Conditions	Complement Titer (centi-milli-liters)	Complement Titer %(more than 8 centi-milli-liters	Pagocytosis %	Pagocytosis Index	Oxidase Activity (optical density of the tests under study)
K	Control....	5.76	6.7	53.87	16.39	0.296
A	Customary .	4.2	7.5	59.4	14.01	0.300
B	"	5.73	13.0	47.4	10.66	0.232
E	"	5.7	30.0	59.0	14.26	0.233
G	"	4.9	10.0	67.71	17.61	0.368
D	"	5.7	0.0	44.4	14.06	0.215
A	Cold	9.75	25.0	54.7	13.75	0.311
B	Cold	7.25	62.0	75.0	12.73	0.160

Table 6 continued.

A	Influence of the centrifuge	5.6	10.0	97.0	26.1	0.320
B	Same	7.7	50.0	58.0	20.02	0.333
A	K_2HPO_4 load.....	7.0	57.0	26.0	5.01	0.278
B	Same	6.6	55.0	38.0	14.20	0.356
A, in its entirety	Customary and stress	5.6	20.0	55.6	13.04	0.287
B, in its entirety	Conditions.....	6.7	40.0	51.64	11.30	0.275

((text continued)): At the end of the chronic experiment it was important to elucidate, also, to what extent the organism of the animals adapted itself to the decontamination of the preservatives that had been introduced. In the case of inoculating the rats with sodium benzoate, which had received only benzoic acid in the course of the chronic experiment, they did not die even in the case of introducing 4 - 5 grams of the preparation in a twenty-four hour period (in the case of LD_{60}, 2 - 2.5 grams to 1 kilogram of body weight). At the same time, in group B, even though in this case, too, the rats received benzoic acid in the course of the chronic experiment, in mixture with sodium bisulfite, this level of endurance, on the part of the animals, of sodium benzoate, did not rise (P is greater than 0.1). In the case of administering to the rats, in the course of days, of 2 - 3 milliliters of a solution of sodium bisulfite, which corresponds to 0.860 - 1.260 milligrams of the preparation (empirically minimal doses, selected by us, which bring about the death of rats); in group K there died 100% of the animals, in group B -- 75%, and in group A -- 50% (in group D no preservative had been introduced). The results of the experiment indicate that the rats do not become accustomed to sodium bisulfite. At the same time, the rats in group A proved to be even more rugged with respect to this substance; and this, to a certain extent, once again under-

scores the presence, in sorbic acid, of nonspecific detoxicating properties relative to other extraneous substances that find their way, along with nutritional products, into the organism. The inoculation of the rats with large doses or sorbic acid (3 - 4 grams to a rat per day) and of nisin (5 - 6 grams to a rat per day) did not lead to the death of the animals and did not even yield any visible signs, whatsoever, of poisoning; this points to the absence of an acute toxicity in these substances.

Following the termination of the chronic experiment, the animals were put to death and their organs were weighed. The most distinct results were obtained with the mice. Thus, in mice belonging to groups B, D and E, in comparison with the mice of groups A and G, there was noted an increased weight of the liver, spleen, testes, and lungs. The lowest liver weight was found in mice belonging to group G. The data are statistically reliable (P was within the range of 0.05). In the rats, there were not observed any changes in the macro- and microscopic structure of the parenchymatous organs.

In relation to the other (remaining) investigations, the following may be noted. Sorbic acid from domestic (Soviet) industrial production proved to be the same nontoxic substance as were the preparations of Czechoslovakian and domestic laboratory production; however because of the admixtures, its biological properties are lower than in the case of the 2 other preparations.

The polymer -- one of the admixtures to the sorbic acid of domestic industrial production -- was not stuedied by anyone prior to us. In its external appearance, it resembles brown-colored coal; it pales upon lying in the light, is tasteless, has an unpleasant, indeterminate odor, dissolves in hydrochloric acid with a change in the color to green; on dissolution in alkali, it does not change color. The chemical composition of the polymer is unknown. The LD_{60} for mice is greater than 4 grams to 1 kilogram of body weight. Thus, the polymer represents a compound of low toxicity. At the same time, there is noted in it the presence of properties of a "paradoxical toxicity" in mice (lassitude, dishevelling of the fur, and others) during the first days of ad-

ministering small doses, with the absence of such symptoms upon the administration of large doses. However, after 10 - 15 days, all the phenomena of toxicosis had passed and, subsequently, the experimental animals did not differ from the control ones. As is known, the essence of similar phenomena has not been studied. It is possible that this points to an active adaptability of the organism to the administration of the substance.

Conclusions

1. A comparative study of the toxicity of 3 specimens of sorbic acid (Czechoslovakian, domestic laboratory and industrial production) has shown that this preservative is nontoxic and possesses favorable biological properties -- it enhances the immunobiological reactivity, the detoxication capacity of the liver, and so forth.

2. The polymer -- the admixture to sorbic acid of domestic industrial production -- in experiments on mice, proved to be a nontoxic substance.

3. English-produced nisin, which had been studied by us, possesses a definite toxicity for mice (a reduction in the survival rate during starvation, even under customary experimental conditions, with an increase in the consumption of feed and water, and also, a gain in body weight on the part of the males).

4. The combined effect of sorbic acid and nisin was manifested as antagonistic as a result of the nonspecific protective effect of sorbic acid: the consumption of water and feed, the body weight, the survival rate closer to the control one than in the groups of animals that had received only nisin.

5. Sodium bisulfite represents a relatively toxic substance, particularly for rats (a reduction in the phagocytic activity of the leucocytes, of the oxidase activity of the blood, of the detoxication capacity of the liver, and so forth).

6. Benzoic acid is of low toxicity for rats and definitely toxic for mice (a reduction in the body weight, and also, in the survival rate, both under customary experimental conditions, as well as in the case of starvation, a re-

duction in the detoxication capacity of the liver, the presence of a cocarcinogenic tendency, and so forth).

7. The combined effect of sodium bisulfite and benzoic acid on the animals has an unfavorable character, in view of the fact that, from the majority of indices applied, both in the case of mice, as well as in the case of rats, data were obtained that were blow the control one (the survival rate, body weight, the complement titer, the phagocytic and oxidase activity, the detoxication capacity of the liver, the carcinogenic tendency, and so forth).

8. From the hygienic point of view, it is expedient to limit the application of sulfurous acid and benzoic acid preservatives as a result of the expansion of application of sorbic acid. Nisin and sulfurous acid preservatives, in the case of an urgent necessity to utilize them, are more practicably used in mixture with sorbic acid.

Bibliography

1. Petrun'kina, A. M., Practical Biochemistry, L(eningrad), 1961, p. 357.

2. Todorov, I., Clinical Laboratory Studies in Pediatrics, Sofia, 1961, p. 606.

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...тание здорового и больного ребенка. М., 1958.—4. Вайль В. С. Методическое посо... по вскармливанию здорового ребенка и расстройствам пищеварения и питания в ...нем детском возрасте. Волгоград, 1958.—5. Небытова-Лукьянчико... М. Н. и др. Полноценное естественное вскармливание ребенка. Л., 1957.—6. Небытова-Лукьянчикова М. Н. Вопр. охр. мат., 1963, № 4, стр. 4.—7. Ведрашко В. Ф. Организация питания детей в детских учреждениях. М., 1961.—8. Олевский М. И. Педиатрия, 1957, № 7, стр. 12; 1961, № 6, стр. 12—9. Хохол Е. Н. Сов. мед., 1957, № 11, стр. 21.—10. Хохол Е. Н., Отт В. Д. Педиатрия, 1963, № 2, стр. 16.—11. Рыскина Е. Б., Харькова Р. М., Шулятьева Е. В. Педиатрия, 1961, № 3, стр. 63.—12. Ведрашко В. Ф. Вопр. охр. мат., 1960, № 5, стр. 3.—13. Барашнева С. М. Вопр. питания, 1963, № 5, стр. 63.—14. Толкачевская Н. Ф. Развитие процессов обмена у детей первого года жизни. М., 1960.—15. Кисляковская В. Г. Обмен азота у детей первого года жизни в зависимости от состава питания. Дисс. канд. М., 1952.

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SOME PRACTICAL PROBLEMS OF EFFECTIVE DIETETICS FOR INFANTS IN THE FIRST YEAR OF THEIR LIFE

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Summary

Under discussion are issues concerning effective feeding of infants in the first year of their life. The observation covered a total of 200 children aged from one to 12 months. The work comprises data consecutive to the study into the liquid supply conditions (drinking regimen) and free feeding, as well as into certain points that concern additional and complementary feeding of nurslings. A pattern of rational feeding is suggested, the efficacy of which finds its confirmation in good development and functional state of infants under observation.

УДК 613.2 : 664.8.035+664.8.035 : 613.2

ЭКСПЕРИМЕНТАЛЬНЫЕ МАТЕРИАЛЫ К ГИГИЕНИЧЕСКОЙ ХАРАКТЕРИСТИКЕ КОМБИНИРОВАННОГО ДЕЙСТВИЯ НЕКОТОРЫХ ХИМИЧЕСКИХ ПИЩЕВЫХ КОНСЕРВАНТОВ

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Задачами нашей экспериментальной работы были следующие: более глубоко изучить и сравнить токсичность сорбиновой кислоты чехословацкого и отечественного (лабораторного и промышленного) производства, а также примеси к последнему препарату полимера; проверить зарубежные литературные данные в отношении отсутствия токсичности низина английского производства (в настоящее время в Советском Союзе проводятся полупроизводственные испытания низина и изучаются возможности получения отечественного препарата); более глубоко исследовать степень выраженности токсических свойств у применяемых в настоящее время консервантов отечественного производства — бисульфита натрия и бензойной кислоты; изучить комбинированное действие на организм животных сорбиновой кислоты с низином и бисульфита натрия с бензойной кислотой.

Работу проводили на чистых препаратах консервантов. Дозы препаратов рассчитывали, исходя из допустимых концентраций их в пищевых продуктах при факторе безопасности (ФБ), равном 1 и 2. Так, сорбиновую и бензойную кислоты вводили в количестве 40 и 80 *мг*, бисульфит натрия — 80 и 160 *мг*, низин — 2 и 4 *мг* на 1 *кг* веса тела животных. Кроме того, низин изучали в дозах 0,4, 40, 400 *мг/кг*, а полимер — в дозах 0,4 и 800 *мг/кг*. Острые опыты не ставили, подострые проводили в ограниченном объеме. Основное внимание уделяли хроническим опытам.

В работе использовано 1900 белых мышей и 400 белых крыс обоего пола и разного веса (согласно литературным данным, при изучении пищевых консервантов другими авторами опыты на мышах не ставились).

В хронических опытах препараты вводили подопытным животным по мет... групповой затравки. Для этого мы готовили затравочные смеси, состоявшие из ис... туемых консервантов и продуктов, входящих в рацион животных. Контрольные жи... ные получали такие же смеси, но без консервантов. Смеси давали отдельно от об... корма.

Статистическую обработку полученных данных проводили по методам вариа... онной статистики. Определяли средние арифметические, квадратичные отклонения ... средних, средние ошибки показателей, ошибки разности показателей, показатели ... щественности (Т, Р, χ^2), коэффициенты корреляции и регрессии в зависимости от це... сообразности применения соответствующих статистических приемов.

Проведено 3 серии исследований. Длительность хронических опытов 8—18 мес... цев, подострых 3 месяца.

При обследовании животных применено более 30 показателей: наблюдение ... общим состоянием, воспроизведение потомства, выживаемость в обычных условия... опыта и при голодании, потребление воды и корма, прибавка в весе тела, проба ... охлаждение организма, физические нагрузки, пробы с четыреххлористым углерод... и K_2HPO_4, определение формулы крови, титра комплемента, фагоцитарной акти... сти лейкоцитов и оксидазной активности крови, РОЭ по Ефману, щелочности кр... и ацетоновых тел в крови, белка в моче. Кроме этого, проводили гистопатологиче... исследования, а также наблюдения по выявлению возможного канцерогенного и ... канцерогенного действия изучаемых консервантов.

Животные были разделены на следующие группы: К — контроль, А — получа... шие смесь сорбиновой кислоты и низина, Б — смесь бисульфита натрия и бен... ной кислоты, В — низин, Г — сорбиновую кислоту, Д — бисульфит натрия и Е ... бензойную кислоту.

Проведенные исследования показали, что наиболее высокий абс... лютный и относительный прирост веса тела, а также потребление в... ды и корма при одновременном снижении выживаемости как в обычны... условиях опыта, так и при голодании отмечались у самцов как мыше... так и крыс, получавших низин (группа В). Сорбиновая кислота ... большинству примененных показателей проявила себя как антагони... низина. Так, в группе А вес тела, выживаемость, потребление воды ... корма были в тех же пределах, что и у контрольных животных, а ... группе Г выживаемость у мышей была даже на 8% выше, чем в кон... роле. У животных, особенно у мышей, получавших бисульфит натри... и бензойную кислоту (раздельно и в смеси) указанные показате... были ниже, чем в контроле. Бензойная кислота оказала отрицательно... влияние на вес тела и выживаемость мышей (к крысам это не относи... лось). Результаты представлены в табл. 1—4.

Таблица 1

Прибавка в весе мышей за 8 месяцев опыта (ФБ-1)

Группа и пол мышей	Исходный вес 8—15 г			Исходный вес 16—25 г		
	абсолютная прибавка		относительная прибавка (в %)	абсолютная прибавка		относительная прибавка (в %)
	в г	в %		в г	в %	
К самцы	26,0	185,0	94,40	10,74	53,50	42,47
К самки	14,16	97,6	68,13	15,20	90,03	68,4[illegible]
А самцы	28,0	257,0	108,60	18,31	94,03	66,37
А самки	19,3	160,0	93,30	13,90	72,02	55,29
Б самцы	26,67	179,0	105,12	15,55	77,60	67,00
Б самки	14,01	108,0	73,67	13,39	72,10	56,14

Из данных, приведенных в табл. 4, видно, что наименьшая выжи... ваемость крыс-самцов при голодании наблюдается в группе В. В сра... нении с группой Г (в контроле не было самцов) эта разница статисти...

Таблица 2

Прибавка в весе крыс за 17 месяцев опыта (ФБ-1)

Группа и пол крыс		Исходный вес (в г)	Абсолютная прибавка		Относительная прибавка (в %)
			в г	в %	
К	самцы	111,88	227,65	203,49	111,04
	самки	101,2	170,8	168,77	98,97
А	самцы	101,79	258,21	253,67	126,39
	самки	97,54	198,0	202,99	110,85
Б	самцы	116,16	191,48	164,84	97,41
	самки	98,40	167,06	151,80	99,42

Таблица 3

Вес тела, потребление корма (брикетов) и воды, выживаемость за опытный период и выживаемость при голодании мышей (ФБ-2)

Группа и пол мышей		Абсолютная прибавка		Относительная прибавка (в %)	Потребление в день		Выживаемость	
		в г	в %		корма (в г)	воды (в мл)	за опытный период (в %)	при голодании (в днях)
К	самцы	16,57	199,39	109,7	2,33	3,67	60	3,86
	самки	12,13	166,83	93,26	2,81	4,44	72	5,22
А	самцы	12,19	128,72	82,74	2,34	3,32	48	10,0
	самки	10,68	114,59	78,35	2,48	4,42	74	5,71
Б	самцы	16,94	174,3	100,9	3,24	3,54	30	3,67
	самки	12,24	140,69	87,85	2,32	2,63	38	4,67
Г	самцы	13,35	145,44	89,78	2,17	3,87	92	4,28
	самки	13,47	144,47	89,36	2,13	3,31	52	4,72
В	самцы	19,20	226,85	118,45	3,36	5,92	12	3,52
	самки	12,47	143,33	87,99	4,31	4,34	32	4,57
Д	самцы	13,30	143,01	88,80	2,43	3,38	70	4,71
	самки	11,21	124,14	80,71	1,98	3,13	68	5,64
Е	самцы	11,28	124,52	80,87	2,57	3,85	68	4,4
	самки	10,95	118,23	78,05	2,65	3,52	68	4,4

чески достоверна и может быть объяснена влиянием консерванта, так как χ^2 для исходного веса и длительности жизни крыс при голодании более 99%.

Опыты с физическими нагрузками проводили в виде плавания по общепринятой методике с добавочным грузом, прикрепленным к хвосту мышей, в размере 5 и 50% к весу тела. Результаты представлены в табл. 5.

Из табл. 5 видно, что наибольшая выносливость к физическим нагрузкам отмечается у самок мышей группы А и Г (при 5% добавочном грузе к весу тела). По сравнению с группами К, Б, Д эта разница статистически достоверна и составляет соответственно $P<0{,}001$, $<0{,}05$, $<0{,}05$. При этом не установлено определенной зависимости между весом тела мышей и их выносливостью к физическим нагрузкам, что позволяет отнести различие в продолжительности удерживания на поверхности воды за счет воздействия на организм изучаемых консервантов.

Таблица 4

Результаты опыта с голоданием крыс (ФБ-2)

Группа и пол крыс		Исходный вес тела (в г)	Потеря веса (в %)	Выживаемость (в днях)	Достоверность (P)
К	самки	179,5	36,21	9,75	>0,05
Г	самцы	225,2	43,7	12,2	
	самки	170,5	38,65	9,5	
Д	самцы	221,2	43,4	11,8	>0,1
	самки	162,4	39,2	8,8	>0,5
Е	самцы	193,6	41,0	12,2	
	самки	176,8	36,2	12,0	
В	самцы	185,5	39,1	9,25	<0,05
	самки	173,0	37,6	9,5	

У 8 из 100 мышей группы Б были обнаружены спонтанные опухоли, а в третьем поколении из 8 мышей у одной, в то время как в других группах этого не отмечено. В связи с полученными данными проведены исследования по выявлению возможного канцерогенного и коканцерогенного действия изучаемых консервантов, а также влияния избыточного веса тела животных на скорость развития введенных внутрибрюшинно клеток асцитного рака Эрлиха. Результаты этих исследований будут опубликованы отдельно. По полученным данным, бензойная кислота и бисульфит натрия, как сами по себе, так и в смеси, в известной мере обладают коканцерогенным действием.

Для определения общей детоксикационной способности печени мы применили нагрузку четыреххлористым углеродом: для мышей 0,1 *мл* и для крыс 0,6 *мл*. Однократное введение его животным через рот показало, что бисульфит натрия, бензойная кислота и низин понизили, а сорбиновая кислота и полимер повысили детоксикационную способность печени. Данные статистически достоверны.

В качестве общего показателя иммунологического состояния подопытных животных мы использовали определение титра комплемента и фагоцитарной активности лейкоцитов; для характеристики уровня окислительных процессов, а также функционального состояния печени — определение оксидазной активности крови.

Определение титра комплемента было проведено по классическому методу при уменьшенных пропорциях взятых реагентов в 10 раз и разведении сыворотки крови в 20 раз. Фагоцитарную активность лейкоцитов определяли также по классической методике с использованием 2-миллиардной взвеси стафилококка штамма № 207. Окси-

дазную активность крови
вина [1]. Обследование живо
подопытных крысах из каждо

Однако в связи с
с целью изучения влия
не стрессовых условий
полнительно разделил
да (в зимнее время) и
ли также нарушение
таты этих исследовани

Из данных, при
табл. 6, видно, что на
кие показатели в групп
обычных условиях опы
нении с группой Б это р
тистически достоверно
для титра комплемента
В группе Д очень низ
тарная активность л
оксидазная активнос
В группе Е показатели
контроля.

Холод и пребыван
фуге более отрицатель
на группу Б (в сравне
пой А), причем снизили
затели. Опыт с воздейс
да, проведенный за 8
этого, дал идентичные
в группе А оксидазная
почти не изменилась
оптической плотности
на 0,030), а в группе
лась на 0,282. В подос
(при ФБ-20) оксидазна
крови повышалась в гр
ше, чем в группе Б (по
тической плотности в
конце опыта; в группе
0,442, в группе Б — 0,
Эти данные указывают
изучавшиеся смеси
влияют на окислительн
в организме животных.
новая кислота в смес
при всех обстоятельств
оксидазную активность
то бисульфит натрия (
дельно) в малых дозах
в обоих случаях являе
данного консерванта.

Изменения, отмеч
ческом опыте позволил
уровне ацетоновых тел
тел методом Нательсон
пе А — 3,4 *мг*% и в гр
ле того, как эти крыс
ФБ-20), при обследова

дазную активность крови определяли с помощью парафенилендиамина по методу Равина [1]. Обследование животных по этим тестам мы проводили в динамике на 10—20 подопытных крысах из каждой группы.

Однако в связи с отсутствием статистически достоверных сдвигов с целью изучения влияния консервантов на животный организм на фоне стрессовых условий в конце хронического опыта всех животных дополнительно разделили на подгруппы и подвергли воздействию холода (в зимнее время) и перегрузке в центробежном барабане, вызывали также нарушение функции почек путем нагрузки K_2HPO_4. Результаты этих исследований представлены в табл. 6.

Из данных, приведенных в табл. 6, видно, что наиболее высокие показатели в группах А и Г в обычных условиях опыта. В сравнении с группой Б это различие статистически достоверно, особенно для титра комплемента ($P<0{,}001$). В группе Д очень низкая фагоцитарная активность лейкоцитов и оксидазная активность крови. В группе Е показатели в пределах контроля.

Таблица 5

Выносливость к физическим нагрузкам у мышей (ФБ-1)

Группа и пол мышей		Время плавания (в мин.) добавочный груз к весу тела 5%	Время плавания (в мин.) добавочный груз к весу тела 50%
К	самцы	4,66	5,0
	самки	5,17	5,5
А	самцы	5,78	27,25
	самки	26,1	6,52
Б	самцы	11,9	16,33
	самки	13,71	13,55
Г	самцы	11,67	—
	самки	24,17	—
Д	самцы	2,6	7,0
	самки	7,54	5,42
Е	самцы	11,71	—
	самки	16,33	—

Холод и пребывание в центрифуге более отрицательно повлияли на группу Б (в сравнении с группой А), причем снизились все показатели. Опыт с воздействием холода, проведенный за 8 месяцев до этого, дал идентичные результаты: в группе А оксидазная активность почти не изменилась (показания оптической плотности понизились на 0,030), а в группе Б она снизилась на 0,282. В подостром опыте (при ФБ-20) оксидазная активность крови повышалась в группе А больше, чем в группе Б (показания оптической плотности в начале и в конце опыта: в группе А — 0,196 и 0,442, в группе Б — 0,161 и 0,623). Эти данные указывают на то, что изучавшиеся смеси консервантов влияют на окислительные процессы в организме животных. Если сорбиновая кислота в смеси с низином при всех обстоятельствах повышает оксидазную активность крови в биологически благоприятных пределах, то бисульфит натрия (как в смеси с бензойной кислотой, так и раздельно) в малых дозах снижает, а в больших резко повышает ее, что в обоих случаях является биологически неблагоприятным свойством данного консерванта.

Изменения, отмеченные в оксидазной активности крови, в хроническом опыте позволили предположить наличие возможных сдвигов в уровне ацетоновых тел в крови. При определении у крыс ацетоновых тел методом Нательсона [2] в группе К обнаружено 2,5 *мг*%, в группе А — 3,4 *мг*% и в группе Б — 1,97 *мг*%. Однако через 2 недели после того, как эти крысы были поставлены на подострый опыт (при ФБ-20), при обследовании на содержание ацетона в крови животных

получены следующие результаты: в группе К — 1,53 *мг*%, в группе А — 2,15 *мг*% и в группе Б — 2,88 *мг*%. Это указывает на то, что увеличение доз сорбиновой кислоты и низина не способствовало повышению уровня ацетоновых тел в крови, в то время как большие дозы бисульфита натрия и бензойной кислоты этот уровень повысили.

Полученные данные подтверждают наше мнение о том, что смесь бисульфита натрия и бензойной кислоты неблагоприятно влияет на окислительные процессы в организме животных. Различия статистически достоверны (P в пределах 0,05).

При нагрузке K_2HPO_4 (до 1000 *мг* на крысу в день) для создания недостаточности почек было установлено, что препараты неорганического фосфора малотоксичны и малопригодны для этих целей. Однако белка в моче крыс группы Б все же было несколько больше (3,3‰), чем в группе А (2,21‰), $P>0,1$.

Таблица 6

Титр комплемента, фагоцитарная активность лейкоцитов и оксидазная активность крови через 17 месяцев от начала опыта у крыс, находящихся в обычных условиях вивария и подвергающихся воздействию стресс-факторов

Группа крыс	Условия опыта	Титр комплемента (в сантимиллилитрах)	Титр комплемента % (более 8 сантимиллилитров)	Фагоцитоз %	Фагоцитоз индекс	Оксидазная активность (оптическая плотность исследуемых проб)
К	Контроль	5,76	6,7	53,87	16,39	0,296
А	Обычные	4,2	7,5	59,4	14,01	0,300
Б	»	5,73	13,0	47,4	10,66	0,232
Е	»	5,7	30,0	59,0	14,26	0,233
Г	»	4,9	10,0	67,71	17,61	0,368
Д	»	5,7	0,0	44,4	14,06	0,215
А	Холод	9,75	25,0	54,7	13,75	0,311
Б	»	7,25	62,0	75,0	12,73	0,160
А	Воздействие центрифуги	5,6	10,0	97,0	26,1	0,320
Б	То же	7,7	50,0	58,0	20,02	0,333
А	Нагрузка K_2HPO_4	7,0	57,0	26,0	5,01	0,278
Б	То же	6,6	55,0	38,0	14,20	0,356
А в целом	Обычные и стрессовые условия	5,6	20,0	55,6	13,04	0,287
Б в целом		6,7	40,0	51,64	11,30	0,275

В конце хронического опыта важно было выяснить также, в какой мере организм животных приспособился к обезвреживанию вводимых консервантов. При затравке бензойнокислым натрием крыс, получавших только бензойную кислоту в течение хронического опыта, они не погибали даже при введении 4—5 *г* препарата в сутки (при LD_{50} 2—2,5 *г* на 1 *кг* веса тела). В то же время в группе Б, несмотря на то что и здесь крысы получали бензойную кислоту в течение хронического опыта, в смеси с бисульфитом натрия этот уровень переносимости животных бензойнокислого натрия не повысился ($P>0,1$). При введении крысам в течение суток 2—3 *мл* раствора бисульфита натрия, что соответствует 0,860—1,260 *мг* препарата (эмпирически подобранные нами минимальные дозы, вызывающие гибель крыс), в группе К погибло 100% животных, в группе Б — 75%, в группе А — 50% (в группе Д консервант не вводили). Результаты опыта показывают, что привыкания к бисульфиту натрия у крыс нет. В то же время крысы группы А оказались даже более выносливыми к этому веществу, что в известной мере еще раз подчеркивает наличие у сорбиновой кислоты неспецифических детоксицирующих свойств по отношению к другим посторонним веществам, попадающим с продуктами питания в организм. Затравка крыс большими дозами сорбиновой кислоты (3—4 *г* на

крысу в сутки) и ни
бели животных и да
равления, что указыв
ществ

После окончан
их органы взвешен
мышах. Так, у мыше
Г отмечен повышен
ший вес печени
верны (P в пределах
микроскопической стр

В отношении про
Сорбиновая кислота о
залась таким же нето
кого и отечественного
биологические свойств

Полимер — одну
го промышленного пр
нему виду он похож
свету, безвкусен, имее
ся в соляной кислоте
в щелочи цвет не из
LD_{50} для мышей более
является малотоксичн
наличие свойств «пар
взъерошенность шерст
отсутствии таковых пр
рез 10—15 дней все я
допытные животные н
ность подобных явлен
тивную приспособляем

1. Сравнительное
кислоты (чехословацк
ного производства) по
дает благоприятными
нобиологическую реак
ни и пр.

2. Полимер — при
мышленного производс
веществом.

3. Изученный нами
деленной токсичностью
данин и в обычных усл
и воды, а также прибав

4. Комбинирование
вилось в антагонизме
сорбиновой кислоты: п
мость ближе к контрол
низин.

5. Бисульфит натр
особенно для крыс (с
оксидазной активности
и пр.).

6. Бензойная кисл
сична для мышей (сн

крысу в сутки) и низина (5—6 *г* на крысу в сутки) не привела к гибели животных и даже не давала каких-либо видимых признаков отравления, что указывает на отсутствие острой токсичности у этих веществ

После окончания хронического опыта животные были забиты и их органы взвешены. Наиболее отчетливые результаты получены на мышах. Так, у мышей групп Б, Д и Е в сравнении с мышами групп А и Г отмечен повышенный вес печени, селезенки, яичек, легких. Наименьший вес печени был у мышей группы Г. Данные статистически достоверны (*P* в пределах 0,05). У крыс не отмечено изменений в макро- и микроскопической структуре паренхиматозных органов.

В отношении прочих исследований можно отметить следующее. Сорбиновая кислота отечественного промышленного производства оказалась таким же нетоксичным веществом, как и препараты чехословацкого и отечественного лабораторного производства, но из-за примесей биологические свойства ее ниже, чем у 2 других препаратов.

Полимер — одну из примесей к сорбиновой кислоте отечественного промышленного производства — до нас никто не изучал. По внешнему виду он похож на уголь бурого цвета, бледнеет при лежании на свету, безвкусен, имеет неприятный неопределенный запах, растворяется в соляной кислоте с изменением цвета до зеленого, при растворении в щелочи цвет не изменяет. Химический состав полимера неизвестен. LD_{50} для мышей более 4 *г* на 1 *кг* веса тела. Таким образом, полимер является малотоксичным соединением. Вместе с тем у него отмечено наличие свойств «парадоксальной токсичности» у мышей (вялость, взъерошенность шерсти и пр.) в первые дни введения малых доз при отсутствии таковых признаков при введении больших доз. Однако через 10—15 дней все явления токсикоза проходили и впоследствии подопытные животные не отличались от контрольных. Как известно, сущность подобных явлений не изучена. Возможно, это указывает на активную приспособляемость организма к введению вещества.

Выводы

1. Сравнительное изучение токсичности 3 образцов сорбиновой кислоты (чехословацкого, отечественного лабораторного и промышленного производства) показало, что этот консервант нетоксичен и обладает благоприятными биологическими свойствами — повышает иммунобиологическую реактивность, детоксикационную способность печени и пр.

2. Полимер — примесь к сорбиновой кислоте отечественного промышленного производства — в опытах на мышах оказался нетоксичным веществом.

3. Изученный нами низин английского производства обладает определенной токсичностью для мышей (снижение выживаемости при голодании и в обычных условиях опыта при увеличении потребления корма и воды, а также прибавка в весе тела у самцов).

4. Комбинированное действие сорбиновой кислоты и низина проявилось в антагонизме за счет неспецифического защитного действия сорбиновой кислоты: потребление воды и корма, вес тела, выживаемость ближе к контролю, чем в группах животных, получавших только низин.

5. Бисульфит натрия является относительно токсичным веществом, особенно для крыс (снижение фагоцитарной активности лейкоцитов, оксидазной активности крови, детоксикационной способности печени и пр.).

6. Бензойная кислота малотоксична для крыс и определенно токсична для мышей (снижение веса тела, а также выживаемости как в

обычных условиях опыта, так и при голодании, снижение детоксикационной способности печени, наличие коканцерогенности и пр.).

7. Комбинированное действие бисульфита натрия и бензойной кислоты на животных имеет неблагоприятный характер, так как по большинству примененных показателей как у мышей, так и у крыс получены данные ниже контроля (выживаемость, вес тела, титр комплемента, фагоцитарная и оксидазная активность, детоксикационная способность печени, канцерогенность и пр.).

8. С гигиенической точки зрения целесообразно ограничить применение сернистокислых и бензойнокислых консервантов за счет расширения применения сорбиновой кислоты. Низин и сернистокислые консерванты при острой необходимости их применения целесообразнее использовать в смеси с сорбиновой кислотой.

ЛИТЕРАТУРА

1. Петрунькина А. М. Практическая биохимия. Л., 1961, стр. 357.—2. Тодоров И. Клинические лабораторные исследования в педиатрии. София, 1961, стр. 606.

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EXPERIMENTAL MATERIALS CONTRIBUTIVE TO HYGIENIC CHARACTERIZATION OF COMBINED EFFECTS PRODUCED BY SOME CHEMICAL FOOD PRESERVANTS

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Summary

Separate and combined toxic effects of sodium bisulfate, benzoic acid, sorbic acid, of a polymer admixed to it, and also of the antibiotic nisin were studied on 1900 mice and 400 rats. The duration of chronic experiments was from 8 to 18 months, and of subacute ones — up to 3 months. Over 30 different characteristic values were tried to specify the action of preservants on the animal organism. The mice, utilized for the first time in studying these substances, turned out to be most sensitive to them.

As shown by tests sorbic acid proved to be nontoxic and was found to exert beneficial biologic action on the animal organism. The admixed to it polymer was nontoxic too. The specimen of nisin put under test tended to increase the consumption of food and water and to augment to body weight, but also reduced the survival rate of mice. The combined action of sorbic acid and nisin resulted in an antagonism, appearing at the expense of the beneficial effect of sorbic acid. Sodium bisulfate proved to be a relatively toxic substance, with benzoic acid being relatively toxic for mice only. According to the author's data the combined effect of sodium bisulfite and benzoic acid was biologically unfavorable for the animals.

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ЗНАЧЕНИЕ ПРОЦЕССОВ ОБЕЗВРЕЖИВАНИЯ В МЕХАНИЗМЕ ИЗБИРАТЕЛЬНОЙ ТОКСИЧНОСТИ ФОСФОРОРГАНИЧЕСКИХ ИНСЕКТИЦИДОВ

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Проблема профилактики неблагоприятного воздействия пестицидов на здоровье населения решается в различных аспектах.

В настоящее время на первый план выступают исследования, направленные на создание пестицидов избирательного действия на вредные организмы при отсутствии такового на полезные.

Генеральное направление в изыскании избирательных пестицидов— это выявление различий в биохимических процессах вредных и по-

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Fd Cosmet. Toxicol. Vol. 8, pp. 369–380. Pergamon Press 1970. Printed in Great Britain

Toxicological Evaluation of some Combinations of Food Preservatives

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Abstract—Short- and long-term studies are reported in which preservatives were administered orally, singly or in pairs, to rats and mice. Daily doses of nisin (2 or 4 mg/kg), sorbic acid (40 or 80 mg/kg), benzoic acid (40 or 80 mg/kg) and sodium bisulphite (80 or 160 mg/kg), and mixtures of benzoic acid/sodium bisulphite (40/80 or 80/160 mg/kg) and sorbic acid/nisin (40/2 or 80/4 mg/kg) were given to mice or rats for 3, 17 or 18 months, and in a further test nisin (0·4–400 mg/kg/day), sorbic acid (40 mg/kg/day) and a polymeric impurity derived from the sorbic acid were tested in mice for 2 months. In addition to the effects of these substances on growth and survival, haematology and blood chemistry (in rats), kidney function (in rats) and reproduction (in mice), the effects of certain stress factors, including dietary restriction, low temperature and carbon tetrachloride administration, on the potential toxicity of the preservatives were studied in both species.

In general, these doses showed some adverse effects in both rats and mice, particularly on growth, survival and susceptibility to stress, but sorbic acid appeared to be the least toxic. The effects of the benzoic acid/sodium bisulphite mixture were generally greater than those of the compounds given singly, while the reverse was the case with the sorbic acid/nisin mixture. The antibiotic, nisin, increased the growth rate when given alone. The authors' recommendation that the use of benzoate and sulphite as preservatives should be further restricted and that sorbic acid should be used instead where possible is being followed in the USSR.

INTRODUCTION

Despite the development of newer techniques, chemical preservatives still retain their economic importance for extending the shelf-life of foods and conserving their nutritional value. Until recently sulphites and benzoates found fairly wide use in the USSR as food preservatives, but now their use is restricted as much as possible. Limited amounts of sulphurous acid are permitted in certain foods, including some wines, jams, fruit candies, starch, tomato puree, dried fruits, fruit juices and some fresh and semi-processed fruits. Benzoic acid is also used, for preserving gelatin, fruit candy and paste, pastilles, margarine, egg mayonnaise, salmon caviare, spiced sprats and some berries and fruit products, and sodium benzoate is permitted in fruit juices, fish preserves and margarine.

Recently sorbic acid and the antibiotic, nisin, have been proposed for extensive use as preservatives, but their safety-in-use had to be established before they could be added to the existing list of permitted food additives. It was decided, therefore, that the technological and economic questions concerning these materials should be studied at the All Union Institute of the Food Canning Industry, while we undertook the toxicological studies.

Toxicological data are available for the administration of each of these compounds individually to experimental animals (Joint FAO/WHO Expert Committee on Food Additives, 1962), but not for their administration in combination. According to the literature, sodium bisulphite fed at dietary levels of 0·05% for up to 2 yr had no adverse effects but levels of 0·1% or higher reduced weight gain and induced histopathological changes in some organs (Fitzhugh, Knudsen & Nelson, 1946). The authors attributed these effects to avitaminosis B_1 following destruction of this vitamin in the diet by the sodium bisulphite. Intubated doses of up to 2·5 g sodium bisulphite given daily to dogs for up to 419 days had no toxic effects. Sodium benzoate had no adverse effects in rats given dietary levels of up to 4% for 90 days, but levels of 8% increased mortality, reduced weight gain and caused hypertrophy of the liver and kidneys (Deuel, Alfin-Slater, Weil & Smyth, 1954). No toxic effects occurred in four generations of rats fed 0·5 or 1·0% in the diet (Kieckebusch & Lang, 1960). High levels of protein in the rat diet have a protective action against this preservative (Lang, 1961). Sorbic acid had no toxic effects when fed in the diet of rats at levels of up to 8% for 90 or 1000 days (Deuel *et al.* 1954; Lang, 1960). No toxic effects were detected in rats fed 0·1–0·3% nisin in the diet, with cheese, for periods of 12 wk–1 yr, survival, weight gain, reproduction indices, haematology, gastro-intestinal and kidney function and organ histology being within the limits of the control animals (Hawley, 1958; Frazer, 1960).

Since mixtures of food additives are ingested in a normal diet, it was considered appropriate to carry out short- and long-term oral toxicity tests in rats and mice on combinations of benzoic acid and sodium bisulphite and of sorbic acid and nisin, as well as on the individual compounds.

EXPERIMENTAL

Materials. Benzoic acid and sodium bisulphite were analytical-grade reagents obtained commercially. Sorbic acid, obtained commercially, contained an unknown polymeric impurity (0·02–0·07% ashed residue) which was insoluble in water, sparingly soluble in acids or alkali and moderately soluble in chloroform. Nisin with an activity of 1×10^6 IU/g was purchased from Aplin & Barrett Ltd., Yeovil, Somerset, UK. It was of largely unknown composition but contained a stabilizer.

Animals. Both sexes of cross-bred white mice and Wistar rats were used, the initial body weights varying according to the nature of the experiment. Mice were housed in groups of 10–25 and rats in groups of five.

Experimental design and conduct

2-Month study in mice. Groups of 25 male and 25 female mice (initial body weight 8–10 g) received for 2 months by oral intubation daily doses of 0·4, 4·0 or 400 mg nisin/kg/day (approximately equivalent to 0·002, 0·02 and 2·0% in human food). Groups of 25 male and 25 female mice (initial body weight 15–20 g) received the same doses of nisin for a similar period. Sorbic acid (40 mg/kg/day) and the polymeric impurity obtained from it (0·8 or 800 mg/kg/day) were given to similar groups of male and female mice from each body-weight range. The mice were observed for general condition and behaviour, and survival, food consumption and weight gain were recorded daily. Tests were carried out to determine the effects of hunger, physical stress and poisoning with carbon tetrachloride on the test animals, compared with untreated controls.

3-Month study in mice. Groups of 50 male and 50 female mice (initial body weight 8–10 g)

received, by oral intubation, sorbic acid or benzoic acid (in doses of 80 mg/kg/day), sodium bisulphite (160 mg/kg/day) or nisin (4 mg/kg/day). Similar groups received mixtures of the same levels of benzoic acid and sodium bisulphite and of sorbic acid and nisin. The observations and tests performed on these mice were the same as those described for the 2-month study, except that a co-carcinogenicity test with Ehrlich ascites carcinoma was added.

17-Month study in mice. Groups of 25 male and 25 female mice (initial body weight 10–15 g) and groups of 25 male and 25 female mice (initial body weight 16–20 g) were given sorbic acid or benzoic acid in doses of 40 mg/kg/day, sodium bisulphite (80 mg/kg/day) or nisin (2 mg/kg/day). Similar groups received mixtures of the same levels of benzoic acid and sodium bisulphite and of sorbic acid and nisin. The test compounds were fed in a paste prior to the main feed, feed and water were provided *ad lib.* and a control group received only the basic diet. The parameters studied on the animals in this test were those listed for the 2-month study, except that poisoning by carbon tetrachloride was not studied and in addition organ weights were recorded. After 8 months on the preservative-combination or control diets some animals were mated and reproduction was studied over five generations. The weight gain of the litters after weaning (at 8–10 g body weight) was recorded for 3·5 months.

18-Month study in rats. Groups of ten male and ten female rats (initial body weight 100–120 g) received sorbic acid or benzoic acid (40 mg/kg/day), sodium bisulphite (80 mg/kg/day) or nisin (2 mg/kg/day). Groups of 50 male and 50 female rats (initial body weight 100–120 g) received sorbic acid (40 mg/kg/day) in combination with nisin (2 mg/kg/day), or benzoic acid (40 mg/kg/day) in combination with sodium bisulphite (80 mg/kg/day). Test compounds were fed in a paste prior to the main feed, while control groups received the basic diet alone. Food and water were provided *ad lib.* Food and water consumption, weight gain and the effects of stress factors were recorded, blood morphology was studied and the following were estimated: titre of serum complement, by our micro-modification of the classical method; phagocytic activity of leucocytes, by the classical method of Kost and Stenco as described by Predtechenskiĭ (1959), serum-ceruloplasmin level by Ravin's method (Petrun'kina, 1959); blood alkalinity (Panisyak, 1952); blood ketones by Natelson's method (Todorov, 1960); blood morphology; erythrocyte sedimentation rate, by a modification of Efmann's method (Mukhorina, 1962); C-reactive protein in serum (Pashinin, 1962).

Effect of stress factors on preservative toxicity in mice and rats

Food restriction. At the termination of the feeding studies, surviving mice were subjected to a restricted dietary intake for up to 5 days, the degree of restriction being 50% after the 2-month, 90% after the 3-month and 100% after the 17-month test. Rats (ten/group) were starved for up to 13 days. Both rats and mice received water *ad lib.* and administration of the preservatives was continued at the same dose levels by oral intubation. Weight loss, mortality rate and, in the case of the rats, the rate of weight-recovery after the return of survivors to a normal diet, were recorded.

Swimming test. All mice surviving at the end of the 2- and 17-month studies were fitted with a 2-g weight on the tail and left to swim in a water bath at 15–18°C. The time until they sank exhausted was recorded.

Low temperature. Groups of 15 rats from those fed combinations of preservatives were exposed to cold (−2–+8°C) for 12–24 hr at the end of the 18-month study.

Centrifugation. To study the functional efficiency of the rat's circulatory–respiratory system

after the prolonged consumption of mixtures of preservatives, groups of 15 rats were subjected to a rotation rate of 90–300 revs/min for 15–30 min.

Carbon tetrachloride detoxication test. This test was carried out to determine the effect of feeding preservatives on the detoxifying action of the liver. Groups of 7–32 mice that had received single preservatives for 2 or 3 months were given carbon tetrachloride by oral intubation in a single dose of 0·1 ml/mouse in sunflower oil (1:1 solution). After receiving one of the combinations of preservatives for 18 months groups of 9–15 rats were treated with orally-intubated carbon tetrachloride either in a single dose of 0·6 ml/rat or in daily doses of 0·2–0·3 ml/rat for 15 days. The mortality rate in these animals was compared with those from the feeding-test control groups given similar doses of solvent.

Kidney function test. Groups of 15 rats that had been given combinations of preservatives were given a kidney function test at the end of the 18-month period of feeding. A saturated solution of K_2HPO_4 was administered in doses of 4 ml/rat twice daily for 6 wk, and haematological studies and urinary protein determinations were carried out.

RESULTS

2-Month study in mice

Weight gain after 15, 30 and 60 days on test showed little divergence from the controls in any group, except in males receiving 0·4, 4·0 or 400 mg nisin/kg/day, in which the weight gain was significantly increased in the group with the lower initial body weight, and in males and females of the group with the higher initial body weight given the polymeric impurity from sorbic acid in a dose of 800 mg/kg. Survival and food consumption in the test groups did not differ significantly from those of the controls. The effect of food restriction on body weight and survival is shown in Table 1. The weight loss was highest in groups receiving the higher doses of nisin and the lower level of polymeric impurity, but survival time was highest in the two groups given the impurity. The same tendency was found in the effects of physical stress and carbon tetrachloride poisoning (Table 2), with mice given nisin being more susceptible than the animals of the other groups, and those given sorbic acid or the lower level of sorbic acid impurity showing a lower degree of mortality than the controls.

3-Month study in mice

Highest mortality rates at 2·5 months were recorded in mice given nisin or the benzoic acid/sodium bisulphite combination (Table 3). Animals given benzoic acid or sodium bisulphite separately showed about twice as high a survival rate as was seen with the combination of the two.

With the exception of males of the nisin group, test animals did not grow as well as the controls at 3 months, but this finding was not associated with a reduced feed intake (Table 4). The weight gain of mice fed the benzoic acid/sodium bisulphite combination was higher than that of mice fed either of the components, whereas in the case of nisin and sorbic acid the reverse was the case.

Under conditions of restricted feed intake mice on benzoic acid, the benzoic acid/sodium bisulphite combination or nisin showed the highest mortality rate (Table 1). The effect of carbon tetrachloride on the mortality of mice fed the preservatives for 3 months can be seen from Table 2. Mice given benzoic acid or sodium bisulphite showed a higher mortality rate than controls but those given sorbic acid were not susceptible to the dose of carbon tetrachloride given. The results of the co-carcinogenicity test are reported below.

Table 1. *Effect of dietary restriction on body weight and survival of preservative-treated mice*

Test compound(s)	Daily intake of preservative (mg/kg)	Group size*	Mortality (%) after 5 days on restricted diet	Weight loss† (%)	Days required to regain lost weight	Survival of fasted animals (days)
50% Food restriction after 2 months on preservative treatment						
None (control)	—	28	43	20		3·37
Nisin	0·4	58	34·5	19·5		3·5
	4·0	54	48·4	24·4		2·7
	400·0	46	70	25·0		3·7
Sorbic	40·0	42	19	14·6		3·5
Sorbic acid impurity	0·8	32	37·5	27·8		5·2
	800·0	28	21·4	20·0		4·25
90% Food restriction after 3 months on preservative treatment						
None (control)	—	16	56·3	41·2		4·6
Benzoic acid	80	14	85·7	31·7		4·3
Sodium bisulphite	160	14	57·2	36·9		4·6
Sorbic acid	80	14	55·7	41·5		4·5
Nisin	4	13	84·6	34·7		4·1
Benzoic acid/sodium bisulphite	80/160	12	83·3	37·9		4·2
Sorbic acid/nisin	80/4	14	64·3	43·8		5·4
100% Food restriction after 17 months on preservative treatment						
None (control)	—	32	12·5	17·8	1·6	
Benzoic acid	40	12	50·0	26·0	2·7	
Sodium bisulphite	80	17	0·0	17·8	2·3	
Sorbic acid	40	10	30·0	25·4	2·1	
Benzoic acid/sodium bisulphite	40/80	33	51·5	22·4	2·6	
Sorbic acid/nisin	40/2	40	7·5	16·7	2·0	

* Values for males and females are pooled together.
Groups of mice were used with body weights in the range 23·5–33·3 g before feed restriction.

Table 2. *Effect of 0·1 ml carbon tetrachloride on the mortality of preservative-treated mice*

Test compound	Daily intake (mg/kg)	Mortality (%)
None (control)*	—	28·6
Benzoic acid*	80	62·5
Sodium bisulphite*	160	45·0
Sorbic acid*	80	0·0
Control†	—	22·3
Nisin†	0·4	46·7
	4·0	56·4
	400·0	70·0
Sorbic acid†	40·0	14·8
Sorbic acid impurity†	0·8	0·0
	800·0	26·6

* Groups of ten mice fed the control or preservative diet for 3 months.
† Groups of 7–32 mice fed the control or preservative diet for 2 months.
The effects of carbon tetrachloride were not tested on mice fed the combinations of preservatives.

Table 3. *Survival rate at 2·5 and 8 months in mice given preservatives orally, alone or in combination*

Test compound(s)	Daily intake (mg/kg)	Survival (%) in Males at month 2·5*	Males 8†	Males 8‡	Females at month 2·5*	Females 8†	Females 8†
None (control)	—	60	72·5	78·5	62	—	—
Benzoic acid	80	68			68		
Sodium bisulphite	160	70			68		
Sorbic acid	80	98			52		
Nisin	4	12			32		
Benzoic acid/sodium bisulphite	80/160	30			38		
	40/80		44·4	35·4		28·5	55·3
Sorbic acid/nisin	80/4	48			74		
	40/2		22·4	9·4		37·9	73·0

* Groups initially comprised 50 male or 50 female mice, weighing 8–10 g in either case.
† Groups initially comprised 9–29 mice of both sexes, weighing 10–15 g. Controls were males only.
‡ Groups initially comprised 26–56 mice of both sexes, weighing 16–25 g. Controls were males only.

17-Month study in mice

Growth and survival. The survival rate of these mice at 8 months can be seen in Table 3. Animals in the control groups had a better survival rate than those receiving the preservative

combinations. The poorest survival was in males receiving sorbic acid in combination with nisin, but this group registered the greatest weight gain as shown in the reproduction study data (Table 5). This was especially marked in the group with initial body weights between 10 and 15 g. The weights of liver, kidney and testes relative to body weight in mice sacrificed at the end of the test period were lower in the group given sorbic acid alone than in all the other groups.

Reproduction study. In the four generations of offspring, the weight gain of the litters was studied for 3·5 months after weaning (at 8–10 g body weight) and the effects of the preservative combinations on the weight of the animals at 3·5 months are shown in Table 5. Those fed sorbic acid in combination with nisin grew better on the whole than mice given benzoic acid in combination with sodium bisulphite.

Effects of stress. In the swimming test a better time was recorded for the groups of mice given sorbic acid alone or in combination with nisin than for mice given benzoic acid alone or in combination with sodium bisulphite. The effect of feed restriction on body weight and survival can be seen in Table 1. Percentage mortality after 5 days of feed restriction was highest in groups receiving benzoic acid alone or in combination with sodium bisulphite. The time required to regain lost weight was also slightly greater in these groups.

Tumour incidence. After the feeding of preservative diets for 17 months, 8/100 mice in the benzoic acid/sodium bisulphite group of the first generation and 1/8 in the third generation of the same group were found to have malignant tumours. No tumours were found in the control or sorbic acid/nisin groups. In connexion with these findings we decided to study the possible co-carcinogenicity of the preservatives using the tumour transplantation test. Erlich ascites mouse carcinoma was transplanted intraperitoneally into mice which had been fed for 3-months on the test diets. The tumour growth was greatest in mice that had received sodium bisulphite, and was less marked in mice given bisulphite in combination with benzoic acid or benzoic acid alone. The tumour growth in mice given sorbic acid alone or in combination with nisin was comparable with that in the control group.

Long-term effects in rats

Rats fed the sorbic acid/nisin mixture grew better than those fed benzoic acid/sodium bisulphite over an 18-month period (Table 6). The titre of serum complement and the phagocytic activity of the leucocytes were also higher in the former than in the latter group, but the level of serum ceruloplasmin was higher in the latter than in the former. Cold stress, centrifugation and kidney loading with K_2HPO_4 had most effect in rats given the benzoic acid/sodium bisulphite combination.

Blood alkalinity, C-reactive protein levels and blood morphology were comparable in all groups including the controls. But the erythrocyte sedimentation rate was increased in rats fed the benzoic acid/sodium bisulphite combination, in which group the level of blood ketones was 1·97 mg/100 ml, compared with 2·5 mg/100 ml in the control group and 3·4 mg/100 ml in the sorbic acid/nisin group.

The results in Table 7 indicate that rats fed benzoic acid for 18 months developed some tolerance to a lethal dose of the compound given terminally. Rats on a sorbic acid/nisin diet tolerated carbon tetrachloride better than did those on the sodium bisulphite/benzoic acid diet. In the latter group, 20% of the animals showed signs of haemorrhagic keratoconjunctivitis, which developed 2 months after the beginning of the study. The effects of starvation (with water available *ad lib.*) were inconclusive, rats in all the test groups surviving longer than the controls.

Table 4. *Mean values of weight gain and daily intake of feed and water in mice given preservatives orally, singly or in combination for 3 months*

Test compound(s)	Daily intake (mg/kg)	Weight gain (% of initial body weight)		Daily feed intake (g)		Daily water intake (ml)	
		Males	Females	Males	Females	Males	Females
None (control)	—	199	167	2·33	2·81	3·67	4·14
Benzoic acid	80	125	118	2·57	2·65	3·85	3·52
Sodium bisulphite	160	143	124	2·43	1·98	3·33	3·13
Sorbic acid	80	145	144	2·17	2·13	3·86	3·31
Nisin	4	227	143	3·36	4·31	5·92	4·34
Benzoic acid/sodium bisulphite	80/160	174	141	3·24	2·32	3·54	2·63
Sorbic acid/nisin	80/4	129	115	2·34	2·48	3·32	4·42

Groups of 50 males and 50 females (initial body weight 8–10 g) were used.
Feed and water consumption were recorded daily throughout the test period.

Table 5. *Weight gain at 3·5 months in four generations of mice given combinations of preservatives from weaning to mating*

Test compounds	Dosage level (mg/kg)	Weight gain (% weight at weaning) at 3·5 months for generation							
		F_1		F_2		F_3		F_4	
		Males	Females	Males	Females	Males	Females	Males	Females
None (control)	—	218	200	147	118	228	212	106	80·5
Benzoic acid/sodium bisulphite	40/80	150	145	103	99	180	217	171	212
Sorbic acid/nisin	40/2	257	202	138	118	219	199	242	187

Table 6. *Mean values of weight gain and daily intake of feed and water in rats fed various preservatives singly or in combination for 18 months*

Test compound(s)	Daily intake mg/kg)	Weight gain at 18 months (% of initial body weight)		Daily feed intake (g)		Daily water intake (ml)	
		Males	Females	Males	Females	Males	Females
None (control)	—	203·49	168·8	17·2	9·6	37·8	26·4
Benzoic acid	40			14·8	13·1	28·6	26·3
Sodium bisulphite	80			14·5	9·8	32·0	25·7
Sorbic acid	40			13·6	9·7	38·7	27·3
Nisin	2			16·8	9·2	39·5	32·6
Benzoic acid/sodium bisulphite	40/80	164·8	151·8				
Sorbic acid/nisin	40/2	253·7	203·0				

Weight gain was estimated in groups of 50 rats (initial body weight 100–120 g) and feed and water intakes in groups of ten.

Table 7. *Effect of carbon tetrachloride and sodium bisulphite and benzoate on mortality in rats fed preservatives singly or in combination for 18 months*

Test compound(s) fed for 18 months	Secondary compound and dose		Mortality (%)
None (control)	Sodium benzoate (mg/kg)	3600	100
Sorbic acid/nisin		3400	71·5
Sodium bisulphite/benzoic acid		3400	100
Benzoic acid		4000	25
None (control)	Sodium bisulphite (mg/kg)	860	100
		430	100
Sorbic acid/nisin		860	66·6
		430	33·3
Sodium bisulphite/benzoic acid		860	100
		430	60
Sorbic acid/nisin	Carbon tetrachloride (ml)	0·6	33·3
		0·2	11·0
Sodium bisulphite/benzoic acid		0·6	60·0
		0·2	27·0

Groups of 3–15 rats were used.

DISCUSSION

The data presented suggest that the preservatives tested had some slight adverse effects on rats or mice in a dosage range equivalent to the maximum levels at which these preservatives may occur in the human diet. The toxic effects of benzoic acid and sodium bisulphite when administered in combination appeared to be synergistic or additive to some extent, while there were indications that the reverse was the case with the sorbic acid/nisin combination. This is an important consideration in the control of the use of preservatives for extending the shelf-life of food for human consumption. In our experiments the sorbic acid showed the lowest toxicity and changes that were recorded in some tests were not considered to be pathological. Moreover, this unsaturated fatty acid has some beneficial effect on the animal organism, being not only a good preservative for food but a nutritional additive with some features of the vitamins. Therefore it was considered reasonable to recommend to the Food Hygiene Department of the Ministry of Health of the USSR a maximum restriction on the permitted list of foods preserved with sodium bisulphite (and other compounds of sulphurous acid) and with benzoates. This recommendation has been accepted.

As the data obtained for nisin were not convincing, it was decided to carry out a further study with nisin manufactured in the Soviet Union. This was recently completed (Shillinger, Bogoroditskaya & Osipova, 1969) and the home-produced nisin was found to be less toxic than the imported. However nisin is an antibiotic, a fact confirmed by our own studies (particularly the finding of weight stimulation in the test animals) and its use as a food preservative should therefore be limited. The high content of nisin occurring naturally in some dairy products is not a sound reason for unrestricted use of this compound as a food preservative, but there is some case for using nisin in combination with sorbic acid, as we have recommended for some products. In the present situation it is considered reasonable to restrict the use of benzoates and sulphites at the expense of extending the use of sorbates, a trend which is at present being followed in the USSR.

REFERENCES

Deuel, H. J., Jr., Alfin-Slater, R., Weil, C. S. & Smyth, H. F., Jr. (1954). Sorbic acid as a fungistatic agent for foods. 1. Harmlessness of sorbic acid as a dietary component. *Fd Res.* **19**, 1.

Fitzhugh, O. G., Knudsen, L. F. & Nelson, A. A. (1946). The chronic toxicity of sulfites. *J. Pharmac. exp. Ther.* **86**, 37.

Frazer, A. C. (1960). Nisin—An assessment of possible biological effect. University of Birmingham, UK.

Hawley, H. B. (1958). Über die Zulässigkeit und Anerkennung von Nisin als Lebensmittelzusatz. *Milchwissenschaft* **13**, 257.

Joint FAO/WHO Expert Committee on Food Additives—Sixth Report (1962). Evaluation of the Toxicity of a Number of Antimicrobials and Antioxidants. *Tech. Rep. Ser. Wld Hlth Org.* **228**.

Kieckebusch, W. u. Lang, K. (1960). Die Verträglichkeit der Benzoesäure im chronischen Fütterungsversuch. *Arzneimittel-Forsch.* **10**, 1001.

Lang, K. (1960). Die Verträglichkeit der Sorbinsäure. *Arzneimittel-Forsch.* **10**, 997.

Lang, K. (1961). Die Notwendigkeit der Verwendung von konservierter Lebensmittel. *Z. Lebensmittelunters. u. -Forsch.* **112**, 341.

Mukherina, K. V. (1962). Eksperimental'nye Materialy o Deistvii na Zhivotnyi Organizm Gidrazida Maleinovoi Kislotý, Zaderzhivayushchego Prorastanie Kartofelya v Protsesse Dlitel'nogo Khranenia. Dissertation, Cand. Med. Sci. Sverdlowsk.

Panisyak, V. I. (1952). Titrometricheskii mikrometod kolichestvennogo opredeleniya shchelochnocti krovi. *Trudý smolensk. gos. med. Inst.* **4**, 52.

Pashinin, I. K. (1962). Opýt Polycheniya Antisývorotki k C-reaktivnomu Belku i Primenenie eé dlya Diagnostiki Vospalitel'nýx Protsessov. Dissertation, Cand. Med. Sci. Leningrad.

Petrun'kina, A. M. (1959). Biokhimicheskie Metodý Issledovanii. Moscow, Medgiz.

Predtechenskii, W. E. (1959). Laboratornye Metodý Issledovaniya. Moscow, Medgiz.

Shillinger, Yu. I., Bogoroditskaya, V. P. & Osipova, I. N. (1969). Gigienicheskaya kharakteristika otechestvennogo preparata nizina-antibiotika dlya konservirovaniya pishchevýkh produktov. *Vop. Pitan.* **22**, 44

Todorov, I. (1960). Laboratornye Metodý Issledovaniya v Pediatrii. Sofia, Bulgaria.

Evaluation toxicologique de certaines combinaisons de produits de conservation des aliments

Résumé—L'article décrit des études à court et à long terme, au cours desquelles des produits de conservation ont été administrés par voie orale, isolément ou par paires, à des rats et à des souris. Des rats ou des souris ont reçu pendant 3, 17 ou 18 mois des doses quotidiennes de nisine (2 ou 4 mg/kg), d'acide sorbique (40 ou 80 mg/kg), d'acide benzoïque (40 ou 80 mg/kg) ou de bisulfite de sodium (80 ou 160 mg/kg) ou de mélanges d'acide benzoïque et de bisulfite de sodium (40/80 ou 80/160 mg/kg) et de mélanges d'acide sorbique et de nisine (40/2 ou 80/4 mg/kg). La nisine (0,4–400 mg/kg/jour), l'acide sorbique (40 mg/kg/jour) et une impureté polymère dérivée de l'acide sorbique ont été testés pendant 2 mois sur des souris au cours d'un autre essai. En plus des effets de ces substances sur la croissance et sur la survie, sur l'hématologie et la chimie du sang (chez les rats), sur la fonction rénale (chez les rats) et sur la reproduction (chez les souris), on a étudié, chez les deux espèces animales, les effets de certains facteurs de stress: restriction alimentaire, basse température et administration de tétrachlorure de carbone, sur la toxicité potentielle des produits.

D'une manière générale, les doses précitées ont eu certains effets nocifs, autant chez les rats que chez les souris, plus particulièrement sur la croissance, la survie et la sensibilité au stress. L'acide sorbique s'est révélé le moins toxique. Les effets du mélange d'acide benzoïque et de bisulfite de sodium étaient généralement plus prononcés que ceux de ces produits administrés séparément, mais l'inverse a été observé pour le mélange d'acide sorbique et de nisine. Administré seul, l'antibiotique qu'est la nisine accélérait la croissance chez les rats. On recommande de restreindre davantage l'emploi du benzoate et du sulfite comme produits de conservation des aliments et de les remplacer autant que possible par l'acide sorbique. Cette recommendation est déjà suivie en URSS.

Toxikologische Prüfung einiger Kombinationen von Konservierungsmitteln für Nahrungsmittel

Zusammenfassung—Es wird über kurz- und langzeitige Untersuchungen berichtet, bei denen Konservierungsmittel einzeln oder paarweise Ratten und Mäusen oral verabreicht wurden. Tägliche Dosen von Nisin (2 oder 4 mg/kg), Sorbinsäure (40 oder 80 mg/kg), Benzoesäure (40 oder 80 mg/kg) und Natriumbisulfit (80 oder 160 mg/kg) und Mischungen von Benzosäure/Natriumbisulfit (40/80 oder 80/160 mg/kg) und Sorbinsäure/Nisin (40/2 oder 80/4 mg/kg) wurden 3, 17 oder 18 Monate Mäusen oder Ratten verabreicht, und bei einem weiteren Versuch wurden 2 Monate lang Nisin (0,4–400 mg/kg/Tag), Sorbinsäure (40 mg/kg/Tag) und eine aus der Sorbinsäure isolierte polymere Verunreinigung an Mäuse verabreicht. Ausser den Wirkungen dieser Substanzen auf das Wachstum und das Überleben, die Hämatologie und Blutchemie (bei Ratten), die Nierenfunktion (bei Ratten) und die Fortpflanzung (bei Mäusen) wurden die Wirkungen bestimmter Stressfaktoren einschliesslich der Futtereinschränkung, der Einwirkung niedriger Temperaturen und der Anwendung von Tetrakohlenstoff auf die mögliche Toxizität der Konservierungsmittel bei beiden Species untersucht.

Im allgemeinen zeigten diese Dosen einige nachteilige Wirkungen bei Ratten und Mäusen, besonders auf das Wachstum, das Überleben und die Stressempfindlichkeit, jedoch schien die Sorbinsäure am wenigsten toxisch zu sein. Die Wirkungen der Benzoesäure/Natriumbisulfit-Mischung war im allgemeinen grösser als die der einzeln verabreichten Verbindungen, während es im Fall der Sorbinsäure/Nisin-Mischung gerade umgekehrt war. Das Antibioticum Nisin erhöhte die Wachstumsgeschwindigkeit bei Einzelverabreichung. Der Autor empfiehlt, die Verwendung von Benzoat und Sulfit als Konservierungsmittel weiter einzuschränken. Stattdessen sollte wie in der UdSSR Sorbinsäure stets verwendet werden, wenn es möglich ist.

TABLE I.—MINIMUM SOLUBILITY PURITY TEST DATA

	Solid-to-Solvent Ratios, Gm./Gm.	Concentrations of Saturated Solutions—Corrected to Same H_2O Content, Gm./Gm.
Recovered from butanol-1	0.0412	0.0346
	0.0852	0.0480
	0.122	0.0592
Recovered from butanol-1, dissolved in water, and recovered by ethanol procedure	0.0382	0.0256
	0.0880	0.0271
	0.145	0.0289

SUMMARY

1. Sodium-5-allyl-5-(1-methylbutyl) barbiturate has been isolated from anhydrous butanol and aqueous ethanol.

2. Minimum solubility purity tests and infrared absorption spectra have shown these materials to differ in their composition with respect to their amido-imidol forms.

3. The two isomeric forms can be interconverted by proper choice of solvents.

4. The displacement of tautomeric equilibrium of properly substituted sodium barbiturates effected by isolation from anhydrous alkanol media may be general.

REFERENCES

(1) (a) Gomez, N., *Anales soc. espan. fis. y quim.*, **29**, 280 (1931); Chem. Abstr., **25**, 4183(1931); (b) Gomez, N., *Rev. acad. cienc. exact. fis.-quim. y nat. Madrid*, **31**, 563 (1934); Chem. Abstr., **29**, 5122(1935); (c) Florence, G., Enseline, J., and Pozzi, M., *Compt. rend.*, **195**, 614(1932); Chem. Abstr., **27**, 229(1933); (d) Stuckey, R. E., *Quart. J. Pharm. and Pharmacol.*, **13**, 312(1940); Chem. Abstr., **35**, 3160(1941); (e) Stuckey, R. E., *ibid.*, **14**, 217(1941); Chem. Abstr., **36**, 705(1942).

(2) Manov, G. C., Schuette, K. E., and Kirk, F. S., *J. Research Natl. Bur. Standards*, **48**, 84(1952).

(3) Morotta, D., and Rosanova, G., *Atti. accad. naz. Lincei*, **15**, 753(1932); Chem. Abstr., **27**, 271(1933).

(4) Arndt, F., Loewe, L., and Ergener, L., *Rev. fac. sci., Univ. Istanbul*, **13A**, 103(1948); Chem. Abstr., **43**, 579 (1949).

(5) Vascautanu, E., and Cernatescu, R., *Bull. sect. sci. acad. roumaine*, **25**, 459(1943); Chem. Abstr., **30**, 3259(1945).

(6) (a) Shonle, H. A., Keltch, A. K., and Swanson, E. E., *J. Am. Chem. Soc.*, **52**, 2110 (1930); (b) Tabern, D. L., and Volwiler, E. H., *ibid.*, **56**, 1139(1934).

(7) Turpley, W., and Yudis, M., *Anal. Chem.*, **25**, 121 (1953).

Preliminary Studies on the Effect of Feeding Sorbic Acid Upon the Growth, Reproduction, and Cellular Metabolism of Albino Rats*

By G. E. DEMAREE, D. W. SJOGREN, B. W. McCASHLAND, and F. P. COSGROVE†

Simple observations on the amounts of food eaten daily indicate that the incorporation of 10% sorbic acid into the diet did not appreciably alter the quantity of food consumed by the test animals. Some of the test animals showed a higher growth rate and a higher ratio of liver weight to body weight than did the controls. The reproductive capacity was found to be normal. No significant differences were observed in the oxygen consumption of liver homogenates of the parent generation; the differences observed in the first generation were significant at the 95% level. The female controls of this group showed the highest rate of oxygen consumption. The rate of oxygen consumption of the liver homogenates from the males and females of this generation treated with 10% sorbic acid were almost identical.

IN A PREVIOUS PUBLICATION from this laboratory it was established that sorbic acid, CH_3—CH=CH—CH=CH—COOH,[1] is a satisfactory fungistatic agent for certain pharmaceutical preparations which are susceptible to mold growth (1). Smith and Rollin (2) also found the acid to be effective in protecting cheeses and cheese products from mold spoilage.

Deuel, *et al.* (3), have reported that sorbic acid is harmless to rats and to dogs when incorporated into the diets to the extent of 5%, however, their data indicate that rats fed a diet containing 8% of the acid exhibited a slight but statistically significant increase in liver weight without signs of micropathology.

In view of these results, it was thought desirable to carry out preliminary studies on the effect of a diet containing 10% sorbic acid upon the growth and reproduction, and upon the metabolism of liver homogenates of two generations of albino rats.

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[1] Supplied by Carbide and Carbon Chemical Co., New York, N. Y.

TABLE I.—EFFECT OF 10% SORBIC ACID DIET ON GROWTH RATE OF ALBINO RATS

Days on Diet	Males: No. of Rats	Control Diet Wt. in Gm. ± $S_{\bar{x}}$	Males: No. of Rats	Sorbic Acid Diet Wt. in Gm. ± $S_{\bar{x}}$	Females: No. of Rats	Control Diet Wt. in Gm. ± $S_{\bar{x}}$	Females: No. of Rats	Sorbic Acid Diet Wt. in Gm. ± $S_{\bar{x}}$
				Parent Generation				
0	5	64.6 ± 1.2	5	64.4 ± 0.8	5	63.6 ± 3.0	5	61.0 ± 1.3
30	5	235.2 ± 6.1	5	212.8 ± 9.9	5	188.8 ± 4.0	5	189.8 ± 8.2
60	5	353.8 ± 29.6	5	342.2 ± 7.5	5	242.6 ± 4.9	5	230.8 ± 10.1
120	5	428.2 ± 9.3	5	430.6 ± 7.9	5	280.4 ± 7.5	5	260.4 ± 9.7
				First Generation				
40	7	311.0 ± 7.8	10	233.7 ± 8.4[a]	7	196.7 ± 6.7	9	179.1 ± 6.7
70	7	356.7 ± 8.4	10	286.2 ± 7.4[a]	7	220.9 ± 5.3	9	205.2 ± 6.8
120	5	414.6 ± 16.8	5	326.8 ± 14.0[a]	5	195.2 ± 13.8	5	219.0 ± 12.8

[a] Significant difference at 99% level.

EXPERIMENTAL

Growth Experiments.—All animals used in this study were bred from Sprague-Dawley stock and were kept in quarters which were maintained at about 25°. The animals were sexed and grouped as: male test rats, male control rats, female test rats, and female control rats. The diet of the control groups was powdered Purina Laboratory Chow and water while the test groups received such chow containing 10% of sorbic acid and water as their diet. Food and water were always available to each rat.

The weights of individual animals of the parent generation were recorded at the beginning of the study and at 30, 60, 90, and 120 days of feeding. The rats used in the second portion of the growth study were selected at random from the young born of the parent generation. The weights of these animals were taken after 40, 70, and 120 days of feeding. Results of the growth studies are shown in Table I.

Reproduction Data.—Animals of the parent generation were mated at ninety days of age and after being fed the respective diets for sixty days while the rats of the first generation were mated after ninety days of age and on these diets for a period of seventy days. All females had young within thirty days. Results are shown in Table II.

Liver Weight and Oxygen Uptake of Liver Homogenates Data.—(*a*) *Preparation of Liver Enzymes.*—The livers were removed from the rats and weighed immediately. In the case of the first generation the animals were selected at random. Results expressed as liver weight percentage body weight are recorded in Table II. The liver enzymes were then prepared according to the method of Lehninger (4) with the following modification: the final suspension was adjusted with phosphate buffer so that 1.0 ml. represented 0.40 Gm. of fresh liver. The preparations were stored at approximately 10° and used within seven hours.

(*b*) *Preparation of Reaction Mixture.*—The main compartment of a 15.0 ml. Warburg flask contained 3.0 ml. of enzyme suspension, 0.10 ml. adenylic acid solution (0.001 *M*), 0.10 ml. magnesium sulfate solution (0.005 *M*), 0.10 ml. phosphate buffer (0.0025 *M*), 0.10 ml. cytochrome C solution (1×10^{-6} *M*), and 0.10 ml. sodium succinate (0.10 *M*).

The side arm contained 0.20 ml. sulfuric acid (1 *N*), and the center well contained 0.10 ml. sodium hydroxide (1 *N*). The reaction times were 30 or 40 minutes at a temperature of 32°. A range of seven to twelve flasks were employed for each liver preparation. Results of oxygen uptake are given in Table III.

TABLE II.—EFFECT OF 10% SORBIC ACID DIET ON REPRODUCTION OF ALBINO RATS[a]

	Parent Generation: Number Mothers	Parent Generation: Mean Number of Offspring ± $S_{\bar{x}}$	First Generation: Number Mothers	First Generation: Mean Number of Offspring ± $S_{\bar{x}}$
Control Diet	5	10.0 ± 2.1	7	8.3 ± 1.8
Sorbic Acid Diet	5	9.4 ± 1.2	9	9.6 ± 1.7

[a] No significant difference found.

DISCUSSION AND SUMMARY

Throughout this study the general appearance and behavior of the animals appeared to be normal. The fact that the rats scattered some of the food made it impractical to report the exact amount of food consumed. Simple observations on the amounts of food eaten daily indicate that the incorporation of 10% sorbic acid into the diet did not appreciably alter the quantity of food consumed by the test animals. This observation is in accordance with the findings of Deuel, *et al.* (3). However, some test animals showed a higher growth rate and a higher ratio of liver weight to body weight than did the controls. Female test rats of the first generation were the only test group not showing an increase in the ratio of liver weight to body weight over the corresponding controls.

While removing the livers it was observed that male test animals of both generations possessed more depot fat than the male controls; however little or no difference was observed between females of the test and the control groups. This may be partially explained by the fact that these rats recently had produced young.

The reproductive capacity of the animals was found to be normal.

There were no significant differences observed in the oxygen consumption of liver homogenates of the parent generation, on the other hand the differences observed in the first generation were significant at the 95% level. The female control homogenates of this group showed the highest rate of oxygen consumption which might be expected because the

TABLE III.—EFFECT OF 10% SORBIC ACID DIET ON LIVER WEIGHT PERCENTAGE BODY WEIGHT AND OXYGEN UPTAKE OF LIVER HOMOGENATES OF ALBINO RATS

	Parent Generation				First Generation			
	Livers from Male Rats		Livers from Female Rats		Livers from Male Rats		Livers from Female Rats	
	Control Diet	Sorbic Acid Diet	Control Diet	Sorbic Acid Diet	Control Diet	Sorbic Acid Diet	Control Diet	Sorbic Acid Diet
Oxygen Uptake[a]	160. ± 6.9	174.5 ± 1.7	186. ± 7.5	166. ± 6.0	73. ± 4.1	100. ± 8.1[b]	177. ± 3.6	90. ± 6.2[b]
% Liver wt. / Body wt.	3.33 ± 0.18	3.92 ± 0.22[c]	4.34 ± 0.50	5.59 ± 0.65[c]	3.18 ± 0.15	3.43 ± 0.59[b]	3.16 ± 0.50	3.41 ± 0.91

[a] Based on μl./40 minutes for the parent generation and μl./30 minutes for the first generation ± standard error.
[b] Significant difference at 95% level.
[c] Significant difference at 99% level.

metabolic rate of female rats is higher than that of males. The rate of oxygen uptake by the male control preparations was the lowest. It is interesting to note that the rate of oxygen consumption of the liver homogenates from both the males and females of this generation treated with 10% sorbic acid were almost identical.

Since the results of the preliminary studies show certain differences, further work is indicated to verify our results. The maximum concentration of sorbic acid in the diet, which will not produce significant changes in the functions of the liver and other organs of several animal species should be determined.

REFERENCES

(1) Puls, D. D., Lindgren, L. F., and Cosgrove, P. P., THIS JOURNAL, 44, 85(1955).
(2) Smith, D. P., and Rollin, N., *Food Research*, 19, 59(1954).
(3) Deuel, H. J., Jr., Alfin-Slater, R., Weil, C. S., and Smyth, H. F., Jr., *ibid.*, 19, 1(1954).
(4) Lehninger, A. L., *J. Biol. Chem.*, 161, 437(1945).

Effect of Glutathione on the Response of *Escherichia coli* to Penicillin*

By ROBERTSON PRATT

Glutathione, in appropriate concentrations, nullifies the slight depressing effect of low concentrations of penicillin (of the order of 4–5 micrograms/ml.) against *Escherichia coli* A.T.C.C. 6522. At higher concentrations of penicillin (up to about 37 micrograms/ml.) addition of glutathione in concentrations from 0.1 to 100 times the molar concentration of the antibiotic seemed to increase the sensitivity of the organism to penicillin.

SEVERAL FACTORS suggest that study of the combined effect of glutathione and penicillin on bacteria might lead to further elucidation of the mechanism of action of the antibiotic. Involvement of —SH groups in the action of penicillin has been suggested frequently (1, 2); interference of penicillin with absorption or utilization of glutamic acid, which constitutes a moiety of the glutathione molecule, has been demonstrated (3–5); structural similarities between penicillin and glutathione have been pointed out (6, 7); and, finally, indirect evidence which suggests that penicillin interferes with the normal equilibrium between the reduced and the oxidized forms of glutathione in sensitive strains of *Micrococcus pyogenes* var. *aureus* has been reported (2, 8, 9, 10).

The present report, which is part of a larger study of different groups of organisms, is concerned with the effect of glutathione on the response of a relatively penicillin-insensitive organism (*Escherichia coli* A.T.C.C. 6522) to the antibiotic in a chemically defined medium.

EXPERIMENTAL

Methods.—*Escherichia coli* A.T.C.C. 6522 was maintained through daily transfers in Anderson's medium as modified by Cowie, *et al.* (11). In this simple medium, containing only dextrose as a carbon source and no organic nitrogen, the organisms multiply somewhat more slowly than in nutrient broth but cultures in the two media have approximately the same absorbance after eighteen hours of

* Received April 25, 1955, from the University of California School of Pharmacy, University of California Medical Center, San Francisco 22.

Tolerability of sorbic acid

by K. Lang

Sorbic acid was approved in the Food Act as a preservative. At our Institute we made in the last years extensive tests on the metabolism and tolerability of sorbic acid. We will give here a review of the tolerability of sorbic acid on the basis of data in the literature and our own tests.

Metabolism

Sorbic acid (trans, trns-DELTA^{2,4}-hexadienic acid) is oxidized in vitro by tissue homogenates or isolated mitochondria in the same manner and at the same rate (or in the case of liver) is converted into acetacetic acid, like other low fatty acids, like butyric acid, capronic acid, octanic acid, (1, 2,3,4). For sorbic acid the same decomposition mechanism was found as for all other fatty acids; activation by binding on coenzyme A (CoA); since already saturated, no dehydration by an acyldehydrogenase, but immediate hydrogenation by crotonase. For the activating enzyme we measured for sorbic acid, compared to other low fatty acids, which the values reproduced in table 1 for the Michaelis constant and reaction value.

Table 1 Michaelis constant and reaction velocity for the "activation of sorbic acid" (4).

Highly purified enzyme from ox liver	KM	reaction in mmole/min/ 14 gamma enzyme at 38 degC
octanate	$1.53 \cdot 10^{-4}$m	$5.75 \cdot 10^{-5}$
heptanate	4.05	1.34
butyrate	1.59	3.57
sorbate	4.0	8.34

The findings obtained in vitro were verified in vivo. Feeding of sorbic acid to starving rats leads a ketosis of the same extent as the feeding of capronic acid, and can be prevented, like the latter, by the administration of glucose. (5) Feeding tests with extremely high doses (see table 3) show clearly that sorbic acid is utilized calorically by the organism.

With extra high doses, a lower-grade omega-oxidation can be demonstrated in analogy to saturated fatty acids with a similar number of C-atoms. After feeding 15 g sorbic acid to a rabbit, 0.20 g muconic acid were isolated from the urine of theanimal.

With chronic feeding of 0.5% sorbic acid in the feed there is no excretion of sorbic acid in the urine (7).

Frequently the suspicion has been voiced that sorbic acid as an acid with conjugated double bonds displays undesirable effects in the organism, particularly as a metabolism antagonist to linoleic acid. An experimental check showed no indication of such an effect of sorbic acid (8). The test was the decrease of the linoleic acid- concentration in the organs of rats on a fat-free diet, with and without doses of sorbic acid. Even 5% sorbic acid in the diet had no effect on the behavior of the linoleic acid concentration or the symptoms of the lack of essential fatty acids. Sorbic acid behaves obviously like the likewise conjugated eleostearic acid, all-trans-DELTA ^{9,11,13} octadecantrienic acid, which is likewise known to act as a metabolic antagonist in the range of the essential fatty acids, and to be utilized in the metabolism (8,9,10).

Inhibition of enzymes or metabolic processes

Sorbic acid, like all low fatty acids, inhibits in low concentrations of 10^{-3} to 10^{-4} m the metabolism (oxidation, formation of acetacetic acid, use for biosyntheses) of other low fatty acids. This is due to the fact that the activated fatty acids compete with each other in low reactions.

The enzymes of the fatty acid activation and of the beta-oxidation are not specified for a single substrate, but for a group of fatty acids. In additional tests the individual substrates must therefore necessarily compete for the enzymes in vitro. These inhibitions show, on the other hand, that sorbic acid has the same metabolism with the participation of the same enzymes as the other fatty acids with a similar number of C-atoms.

Whitaker (10b) has found that sorbic acid inhibits alcohol dehydrogenase.

A 50% inhibition requires a sorbic acid concentration of $6.5 \cdot 10^{-3}$ m. Furthermore he demonstrated the inhibition of ficin, a 50% inhibition requiring $5\text{-}6 \cdot 10^{-4m}$. Not inhibited according to the tests by Whitaker were urease and aldolase.

Completely non-specific inhibiting effects of high sorbic acid concentrations of 10^{-2} and more were not taken into consideration in this table, since they are of no physiological or toxicological interest.

Acute toxicity

The acute toxicity of sorbic acid is very low and is of the order of the saturated fatty acids with the same or a similar number of C-atoms. The present data on the LD/50 of sorbic acid are compiled in table 2.

Table 2 LD/50 of sorbic acid in the rat. All values in g/kg body weight with peroral administration

		g/kg
Well-fed rat	sorbic acid (8)	10.50 ± 1.96
	sodium sorbate calc. as sorbic acid (8)	5.14
	sorbic acid (2)	7.40
	sodium sorbate (2)	7.20
starving rats	male (8)	4.30
	female (8)	3.65

For comparison we list here the data for the LD/50 determined for other low fatty acids: acetic acid 3.3 - 3.5 g/kg; butyric acid 2.9 to 8.8 g/kg and caproicacid 3.0 - 6.8 g/kg rate (per os).

Chronic feeding tests with sorbic acid

Feeding of 40% sorbic acid of 4% caproic acid to dogs for 90 days (total 9 animals), had no effect on the growth, well-being, blood-formation and histological picture of the organs (8). The feeding test made so far on rats are compiled in table 3.

The long-term and most extensive test (700 animals, duration of test so far: 1000 days is still underway at the institute of the author. So far doses of sorbic acid of 0.1% 0.5% and 5% in the feed (corresponding to daily

doses of 15.75 and 750 mg per rat) showed no harmful effect on growth, feed utilization, life expectancy and cause of death. The data obtained for growth, feed utilization, life expectancy and propagation are compiled in tables 4,5 and 6. In the male rats fed with 5% sorbic acid there was a temporary, statistically ascertained acceleration of the growth. It is probably due to the increase of the calorie supply, which is important with 5% sorbic acid in the feed, at a constant feed intake. Furthermore the motor behavior of the rats fed with 0.1% and 0.5% sorbic acid was investigated. No difference was found, compared to the controls.

Table 3 Long-term feeding tests with sorbic acid

4% sorbic acid in feed	animals p.group	test period days	effect	author
10	10-15	120	in the first generation no effect on growth and propagation. In the second generation retarded growth of males not females. Test can not be evaluated, since no data are available on the feed intake. In the first generation in males and females somewhat greater liver weights (calc. as % of body weight), not in the second generation. In the first generation no difference in succinate oxidation of the liver homogenates, in the second generation conspicuously high values in the female controls, which is completely outside the test pattern.	(11)
0.5, 1.0 2.0,4.0 and 8.0	10	90	No difference in growth, feed efficiency, liver- and kidney weights, compared to controls, with the exception of slightly higher liver weights with 8% sorbic acid in the feed. No pathological histological findings. Same results in two parallel tests at two different laboratories.	(8)
0.1 and 0.5	100	1000 so far	No difference in growth, propagation, health, life-expectancy, cause of death, compared to controls.	(7)
5	100	450 so far	In two generations no difference in growth and propagation	(7)

Table 4 Feeding tests with sorbic acid. In each group 100 animals (50 females and 50 males)

	controls	0.1%sorbic	0.5%sorbic	1st generation		2nd generation	
				contr.	5% sorbic acid	controls	5% sorbic acid
Males							
weight increase g							
in 2 weeks	31	35	34	33± 0.8	31±0.9	33± 0.4	33±0.4
4	53	53	54	54± 1.2	65±1.2	55± 1.1	57±1.7
6	90	87	87	88± 1.2	105±1.7	89±1.5	97±2.5
8	114	114	114	112±2.4	136±3.5	117±1.8	129±3.2
feed intake g							
in 2 weeks	102	101	100	110	109	110	106
4	245	245	240	251	251	266	237
6	425	427	425	435	437	440	408
8	602	597	617	596	629	630	594
protein efficiency							
in 2 weeks	1.44	1.55	1.54	1.38	1.44	1.43	1.55
4	1.03	1.03	1.06	1.03	1.25	1.02	1.23
6	1.00	0.97	0.97	0.88	1.21	1.00	1.22
8	0.90	0.91	0.88	0.83	1.09	0.93	1.12
Females							
weight increase in g							
in 2 weeks	30	32	36	30	28	24	27
4	49	50	58	51	62	46	48
6	84	81	86	75	94	74	78
8	101	98	104	97	110	95	101
feed intake in g							
in 2 weeks	102	106	100	110	110	107	102
4	247	251	245	254	255	237	232
6	428	436	424	435	428	405	394
8	601	607	618	596	615	572	567
protein efficiency							
in 2 weeks	1.39	1,43	1.70	1.38	1.28	1.07	1.38
4	0.94	0.95	1.12	1.03	1.23	0.98	1.05
6	0.93	0.89	0.96	0.88	1.11	0.91	1.03
8	0.80	0.77	0.80	0.83	0.90	0.83	0.93

In the framed values the difference with regard to the controls has been statistically ascertained.

The provosional histological examination of 30 animals of the 2nd generation, which received for 252 days 5% sorbic acid, as well as of 30 control animals showed no pathological findings.

Table 5 Life expectancy of animals fed with sorbic acid

	Number of animals with a life of		
	under 600 days	600-900 days	over 900 days
controls	14	46	40
0.1 % sorbic acid	24	33	43
0.5 % "	20	42	38

Table 6 Propagation of rats fed with sorbic acid

I Test groups with 0.1% and 0.5% sorbic acid in the feed

	controls	0.1% sorbic acid	0.5% sorbic acid
number of females	50	50	50
I. coupling (12/4/1957)			
number of all young rats	144	200	216
% of young rats surviving 21 days	73	86	84
II. coupling (3/23/1958)			
number of all young rats	387	427	423
% of young rats surviving 21 days	50	58	44
III coupling (9/10/58)			
Number of all young rats	316	326	297
% of young rats surviving 21 days	54	48	33

II Test group with 5% sorbic acid in the feed

	controls	5% sorbic acid
number of females	50	50
% of females with dealyed puberty	6	2
% of females sterile	8	4
total number of female rats	422	428
% of young rats surviving 21 days	48	46

On the basis of our observations so far 5% sorbic acid in the feed is tolerated without symptoms. With 8% sorbic acid, Deuel and coworkers (8) found a slight enlargement of the liver, but no change in the histological findings. They attributed no pathological significance to their findings. Perhaps the enlarged liver must be interpreted in the sence of a working hypertrophy. If it is considered an incipient damage, which is by no means proven, however, the presumed toxic limiting dose is under 8% and above 5% sorbic acid in the feed.

When feeding 5% sorbic acid in the feed, the daily sorbic acid intake for a rat of about 300 g is 750 mg. Converted to a man of 70 kg, this means a daily dose of 174 g.

Summaries in German and English.

(Translated by Carl Demrick Associates,Inc/IE/t)

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Die Enzyme der Fettsäure-Aktivierung und der β-Oxydation sind nämlich nicht für ein einziges Substrat spezifiziert, sondern jeweils für eine Gruppe von Fettsäuren. Daher muß es zwangsläufig in Zusatzversuchen in vitro zu einer Konkurrenz der einzelnen Substrate um die Enzyme kommen. Diese Hemmungen zeigen andererseits, daß Sorbinsäure denselben Stoffwechsel unter Beteiligung derselben Enzyme wie die anderen Fettsäuren einer ähnlichen C-Atomzahl hat.

Whitaker [10b] hat festgestellt, daß Sorbinsäure Alkoholdehydrogenase hemmt. Eine 50%ige Hemmung benötigt eine Sorbinsäurekonzentration von $6.5 \cdot 10^{-3}$m. Weiterhin wies er eine Hemmung von Ficin nach, wobei die 50%ige Hemmung $5-6 \cdot 10^{-3}$ m benötigt. Nicht gehemmt werden nach den Untersuchungen von Whitaker Urease und Aldolase.

Nicht berücksichtigt in dieser Aufstellung wurden gänzlich unspezifische Hemmwirkungen hoher Sorbinsäure-Konzentrationen von 10^{-2} und mehr, da sie kein physiologisches oder toxikologisches Interesse haben.

Akute Toxizität

Die akute Toxizität der Sorbinsäure ist sehr gering und in der Größenordnung der gesättigten Fettsäuren derselben oder ähnlichen C-Atomzahl gelegen. Die vorliegenden Angaben über die LD_{50} der Sorbinsäure sind in Tab. 2 wiedergegeben.

Tab. 2: LD_{50} der Sorbinsäure bei der Ratte. Alle Zahlen in g/kg Körpergewicht bei Gabe per os.

		g/kg
Gut ernährte Ratte	Sorbinsäure [8]	10,90 ± 1,96
Gut ernährte Ratte	Natriumsorbat (berechnet als Sorbinsäure) [6]	5,14
Gut ernährte Ratte	Sorbinsäure [2]	7,40
Gut ernährte Ratte	Natriumsorbat [2]	7,20
Hungernde Ratten	Männchen [8]	4,30
Hungernde Ratten	Weibchen [8]	5,65

Zum Vergleich seien die für die LD_{50} ermittelten Daten [10a] für andere niedere Fettsäuren wiedergegeben: Essigsäure 3,3—3,5 g/kg, Buttersäure 2,9 bis 8,8 g/kg und Capronsäure 3.0—6.8 g/kg Ratte (per os).

Chronische Fütterungsversuche mit Sorbinsäure

90 Tage lange Verfütterung von 4% Sorbinsäure oder 4% Capronsäure an Hunde (insgesamt 9 Tiere) hatte keinen Einfluß auf Wachstum, Wohlbefinden, Blutbildung und histologisches Bild der Organe [8]. Die bisher an Ratten durchgeführten Fütterungsversuche sind in Tab. 3 zusammengestellt.

Der langfristige und umfangreichste Versuch (700 Tiere, bisherige Versuchsdauer 1000 Tage) läuft gegenwärtig noch im Institut des Verfassers. Er hat bisher bei Dosierungen der Sorbinsäure von 0.1%, 0.5% und 5% im Futter (entsprechend Tagesdosen von 15, 75 und 750 mg je Ratte) hinsichtlich Wachstum, Fortpflanzung, Lebensdauer und Todesursache keinen nachteiligen Befund ergeben. Die erhaltenen Daten für Wachstum, Futterverwertung, Lebensdauer und Fortpflanzung sind in den Tab. 4, 5 und 6 wiedergegeben. Bei den mit 5% Sorbinsäure gefütterten Männchen ergab sich eine vorübergehende statistisch gesicherte Beschleunigung des Wachstums. Sie ist vermutlich durch die bei 5% Sorbinsäure im Futter ins Gewicht fallende Vergrößerung der Kalorienzufuhr bei gleichbleibender Futteraufnahme bedingt. Weiterhin wurde noch das motorische Verhalten der mit 0.1% und 0.5% Sorbinsäure gefütterten Ratten untersucht. Es ergab sich kein Unterschied gegenüber den Kontrollen. Die Untersuchung des Harns ergab keine Ausscheidung von Sorbinsäure.

Die vorläufige histologische Untersuchung von 30 Tieren der 2. Generation, die 252 Tage hindurch 5%

Tab. 3: Langfristige Fütterungsversuche mit Sorbinsäure

% Sorbinsäure im Futter	Tiere je Gruppe	Versuchsdauer Tage	Wirkung	Autor
10	10—15	120	In 1. Generation kein Einfluß auf Wachstum und Fortpflanzung. In 2. Generation Wachstumsverzögerung der Männchen, nicht Weibchen. Versuch ist nicht auswertbar, da keine Daten über den Futterverzehr vorliegen. In 1. Generation bei Männchen und Weibchen etwas größere Lebergewichte (berechnet als % des KG), nicht in 2. Generation. In 1. Generation kein Unterschied in Succinatoxydation der Leberhomogenate, in 2. Generation auffallend hoher Wert bei den weiblichen Kontrollen, der ganz aus dem Versuchsrahmen fällt.	[11]
0,5 1,0 2,0 4,0 und 9,0	10	90	Kein Unterschied in Wachstum, Futter-Efficiency, Leber- und Nierengewichte gegenüber Kontrollen, mit Ausnahme etwas vergrößerter Lebergewichte bei 9% Sorbinsäure im Futter. Kein abnormer histologischer Befund. Gleiche Ergebnisse in 2 Parallelversuchen in zwei verschiedenen Laboratorien.	[8]
0,1 und 0,5	100	z. Z. 1000	Kein Unterschied in Wachstum, Fortpflanzung, Gesundheitszustand, Lebensdauer, Todesursache gegenüber Kontrollen.	[7]
5	100	z. Z. 450	In zwei Generationen kein Unterschied in Wachstum und Fortpflanzung.	[7]

Tab. 4: Fütterungsversuche mit Sorbinsäure. In jeder Gruppe 100 Tiere (50 Weibchen und 50 Männchen).

	Kontrolle	0,1% Sorbinsäure	0,5% Sorbinsäure	I. Generation Kontrolle	I. Generation 5% Sorbinsäure	II. Generation Kontrolle	II. Generation 5% Sorbinsäure
Männchen							
Gewichtszunahme g							
in 2 Wochen	31	33	34	33 ± 0,8	31 ± 0,9	33 ± 0,4	33 ± 0,4
in 4 Wochen	53	53	54	54 ± 1,2	44 ± 1,2	55 ± 1,1	57 ± 1,7
in 6 Wochen	90	87	87	88 ± 1,2	105 ± 1,7	99 ± 1,5	97 ± 2,5
in 8 Wochen	114	114	114	112 ± 2,4	136 ± 3,5	117 ± 1,8	129 ± 3,2
Futteraufnahme g							
in 2 Wochen	102	101	100	110	109	110	106
in 4 Wochen	245	243	240	251	259	266	257
in 6 Wochen	425	427	425	435	437	440	408
in 8 Wochen	603	597	617	596	629	650	594
Protein-Efficiency							
in 2 Wochen	1,44	1,55	1,54	1,38	1,44	1,43	1,55
in 4 Wochen	1,05	1,03	1,06	1,05	1,25	1,02	1,25
in 6 Wochen	1,00	0,97	0,97	0,88	1,21	1,00	1,22
in 8 Wochen	0,90	0,91	0,88	0,85	1,09	0,95	1,12
Weibchen							
Gewichtszunahme g							
in 2 Wochen	50	52	56	50	28	24	27
in 4 Wochen	49	50	58	51	62	46	48
in 6 Wochen	84	81	86	75	94	74	78
in 8 Wochen	101	98	104	97	110	95	101
Futteraufnahme g							
in 2 Wochen	102	106	100	110	110	107	102
in 4 Wochen	247	251	245	274	235	237	232
in 6 Wochen	428	436	424	435	428	405	394
in 8 Wochen	601	607	618	596	615	572	567
Protein-Efficiency							
in 2 Wochen	1,39	1,43	1,70	1,38	1,28	1,07	1,38
in 4 Wochen	0,94	0,95	1,12	1,05	1,25	0,88	1,05
in 6 Wochen	0,93	0,89	0,96	0,88	1,11	0,91	1,05
in 8 Wochen	0,80	0,77	0,80	0,85	0,98	0,85	0,95

Bei den eingerahmten Zahlen ist der Unterschied gegenüber den Kontrollen statistisch gesichert.

Tab. 5: Lebensdauer der mit Sorbinsäure gefütterten Tiere

	Zahl der Tiere mit einer Lebensdauer von		
	unter 600 Tag.	600—900 Tagen	über 900 Tagen
Kontrolle	14	46	40
0,1% Sorbinsäure	24	33	43
0,9% Sorbinsäure	20	43	36

Tab. 6: Fortpflanzung von Ratten bei Verfütterung von Sorbinsäure.

I. Versuchsgruppen mit 0,1% und 0,9% Sorbinsäure im Futter

	Kontrolle	0,1% Sorbinsäure	0,9% Sorbinsäure
Zahl der Weibchen	50	50	50
I. Paarung (4. 12. 57)			
Zahl aller Jungen	144	200	216
% der Jungen, 21 Tage überlebend	73	86	84
II. Paarung (23. 4. 58)			
Zahl aller Jungen	387	427	423
% der Jungen, 21 Tage überlebend	58	58	44
III. Paarung (10. 9. 58)			
Zahl aller Jungen	316	326	297
% der Jungen, 21 Tage überlebend	54	48	33

II. Versuchsgruppe mit 5% Sorbinsäure im Futter (1. Generation)

	Kontrolle	5% Sorbinsäure
Zahl der Weibchen	50	50
% der Weibchen mit verzögerter Geschlechtsreife	6	2
% der Weibchen steril	8	4
Gesamtzahl der Jungen	422	428
% der Jungen, 21 Tage überlebend	49	46

Sorbinsäure verfüttert bekommen hatten, sowie von 30 Kontrolltieren ergab keine abnormen Befunde.

Auf Grund unserer bisherigen Beobachtungen werden 5% Sorbinsäure im Futter symptomlos vertragen. Bei 8% Sorbinsäure fanden Deuel u. Mitarb. [8] eine geringe Vergrößerung der Leber, jedoch ohne Veränderungen des histologischen Bildes. Sie messen ihrem Befund daher keine pathologische Bedeutung zu. Vielleicht ist die Lebervergrößerung im Sinne einer Arbeitshypertrophie zu deuten. Faßt man sie als beginnende Schädigung auf, was jedoch keineswegs erwiesen ist, so liegt die vermutliche toxische Grenzdosis unter 8% und über 5% Sorbinsäure im Futter.

Bei Verfütterung von 5% Sorbinsäure im Futter beträgt die tägliche Sorbinsäure-Aufnahme für eine etwa 300 g schwere Ratte 750 mg. Umgerechnet auf einen 70 kg schweren Menschen ergäbe dies eine Tagesdosis von 174 g.

Zusammenfassung

Sorbinsäure wird im Stoffwechsel wie die gesättigte oder einfach ungesättigte Fettsäure derselben C-Atomzahl in qualitativer und quantitativer Hinsicht umgesetzt und im Organismus kalorisch verwertet. Die in der Sorbinsäure vorhandenen konjugierten Doppelbindungen bedingen im Organismus keine Sonderwirkungen.

Chronische Fütterungsversuche haben ergeben, daß 5% Sorbinsäure im Futter von Ratten sich nicht schädlich auswirken. Wahrscheinlich ist die unschädliche Dosis sogar noch erheblich größer.

Summary

Tolerability of Sorbinic Acid

Qualitative and quantitative transformation of sorbinic acid in metabolismand its caloric utilization in the organism is performed in the same manner as in saturated or in single unsaturated fatty acids containing the same number of C atoms.

The conjugated double bonds in sorbinic acid do not entail special metabolic effects.

Chronic feeding experiments have shown a 5% sorbinic acid content in the food of rats to be innocuous. The tolerated dose is probably even higher.

Literatur

[1] Cohen, P. P., J. biol. Chem. 119, 135 (1939)
[2] Witter, R. F., Newcomb, E. H. u. Stotz, E., J. biol. Chem. 185, 537 (1950); 195, 663 (1952); 200, 705 (1953)
[3] Lang, K. u. Bässler, K. H., unveröffentlicht
[4] Mahler, H. R., Wakil, S. u. Bock, R. M., J. biol. Chem. 206, 453 (1954)
[5] Deuel, H. J. jr., Calbert, C. E., Anisfeld, L., McKeehan, H. u. Blunden, H. D., Food Res. 19, 13 (1954)
[6] Kuhn, R., Köhler, F. u. Köhler, L., Z. physiol. Chem. 247, 197 (1937)
Versuche
[7] Kieckebusch, W. u. Lang, K., Arzneim.-Forsch. 10, 1001 (1960)
[8] Deuel, H. J. jr., Alfin-Slater, R., Weil, C. S. u. Smyth, H. F. jr., Food Res. 19, 1 (1954)
[9] Miller, E. S. u. Burr, G. O., Proc. Soc. exp. Biol. Med. 36, 726 (1937)
[10] Reiser, R., Arch. Biochem. 32, 113 (1951)
[10a] Spector, W. S., „Handbook of Toxicology". Philadelphia und London. Band I. 1956.
[10b] Whitaker, J. R., Food Res. 24, 37 (1959)
[11] Demaree, G. E., Sjogren, D. W., McCashland, B. W. u. Cosgrove, E. P., J. Amer. pharm. Assoc. Sci. Ed. 44, 619 (1955)

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Die Verträglichkeit der p-Chlorbenzoesäure

Von W. Kieckebusch, W. Griem u. K. Lang

p-Chlorbenzoesäure verhält sich im Stoffwechsel analog der Benzoesäure. Hauptstoffwechselprodukt ist p-Chlorhippursäure. Vor einiger Zeit haben wir über Stoffwechselversuche mit ^{14}C-p-Chlorbenzoesäure (in der Carboxylgruppe markiert) an Ratten nach ip. Injektion der Substanz berichtet [2]. p-Chlorbenzoesäure wird innerhalb von 24 h praktisch quantitativ im Harn ausgeschieden. In den Organen wurden nur äußerst geringe Bruchteile (10^{-2} bis 10^{-4}%) der verabreichten Dosis gefunden. Hecht [1], der Stoffwechselversuche mit unmarkierter p-Chlorbenzoesäure angestellt hatte, konnte ebenfalls keine Speicherung der Substanz im Organismus nachweisen.

Wührer [3] verfütterte an einen Hund 12 Monate hindurch pro Tag je 3 g und an 2 Hunde 20 Monate je 1,5 g p-Chlorbenzoesäure, ohne daß eine schädliche Wirkung nachzuweisen war. Auch die histologische Untersuchung der Organe ergab keinen abnormen Befund. Insbesondere wurden keine Veränderungen der Magen- und Darmschleimhaut gefunden.

Da wir sonst in der Literatur keine Angaben über die chronische Toxizität der p-Chlorbenzoesäure fanden, haben wir einen 5 Monate dauernden Fütterungsversuch an Ratten durchgeführt, über den im folgenden berichtet wird.

Methodik

1. Fütterungsversuch

Als Versuchstiere dienten 120 junge Ratten (Elberfelder-Stamm) mit einem Anfangsgewicht von 40—50 g. Sie wurden in drei Gruppen geteilt; jede Gruppe umfaßte 20 Männchen und 20 Weibchen. Die erste Gruppe erhielt keine p-Chlorbenzoesäure und diente als Kontrolle. Die zweite Gruppe erhielt 0,1%, die dritte 0,2% des Futtergewichtes an p-Chlorbenzoesäure im Futter. Das entspricht rund einer Tagesaufnahme von 13 bzw. 26 mg p-Chlorbenzoesäure. Insgesamt erhielt jedes Tier während der Versuchszeit rund 2,0 bzw. 4,0 g p-Chlorbenzoesäure. Die verfütterte Diät war die käufliche Latz-Standardkost. Wiederholte Analysen ergaben einen Proteingehalt der Diät von 22,5% (N×6,25) und einen Wassergehalt von 8,4%. Die Futteraufnahme wurde durch tägliche Einwaage und wöchentliche Rückwaage der gesammelten, nicht verzehrten Reste bestimmt. Die Fütterung erfolgte nach der „paired-feed"-Technik.

Zur Beobachtung der Fortpflanzung wurden die Tiere 8 Wochen nach Versuchsbeginn gepaart. Zur Feststellung einer verzögerten Erreichung der Geschlechtsreife und Kontrolle auf Sterilität erfolgte 2 Monate später eine zweite Paarung.

2. Harnuntersuchungen

Der Harn der Tiere wurde auf Eiweiß und Zucker gegen Ende der Fütterungsperiode untersucht. Weiterhin wurde das Harnsediment mikroskopisch untersucht. Zu diesen Untersuchungen wurden die Tiere in Stoffwechselkäfige für 24 h gesetzt und erhielten während dieser Zeit kein Futter, um jegliche Verunreinigung des Harns durch Futterspuren zu vermeiden.

LUITPOLDWERK MÜNCHEN

Effect of preservatives on the utilization of carotene

by M. Kramer and R. Tarjan

(received on Nov. 27, 1961)

In the winter months a certain part of the vitamin-A need of the population is covered by the carotene content of some preserved vegetables and fruits, where standardized preservatives have been used for their production.

As known, preservatives have an inhibiting effect on the activity of the various enzymes (1) and can therefore also influence the various metabolic processes. It must therefore be assumed that they are not indifferent with regard to the utilization of carotene. According to the literature available to us, the effect of preservatives on the utilization of carotene does not seem to have been studied so far. But since this question is of practical importance, we intend to investigate the various natural diet factors (2,3) and the metal impurities in the foodstuffs (4) and to determine theinfluence, if any, of the preservatives which are approved in Hungary and used by the industry, on the utilization of carbtene.

Experimental part

For the clarification of this question we made the following animal tests:

a) After the administration of amounts of preservatives which did not influence the feed consumption and the growth of the test animals and after the feeding of certain carotene doses, we determined the vitamin-A content in the liver and kidney and it compared with the vitamin-A deposit of the control animals.

b) With preservatives which do influence the utilization of carotene, we repeated the test with smaller doses and observed subsequently the effect after prolonged feeding.

C) In order to clarify the mechanism of action, we investigated the extent of the carotene excretion in the administration of various preservatives, so that any effect on the carotene resorption and its extent can be determined.

1. In the first test series 40 white male Wister rats, which were 8 weeks

old, were divided into four groups of 10 animals each. As a basic diet the animals received the grain mixture used in our previous tests to which casein had been added and which contained carotene only in traces (5). To the dry feed of the first group were added 2.4% formic acid, to that of the second group 2.0 sorbic acid, and to that of the third group 1.5 sodium benzoate. The fourth group served as a control. Formic acid and sodium benzoate were added in the form of a 10% aqueous solution to the feed. For dissolving the sorbic acid we used KOH in an amount which brought the solution to a pH of 7. The feed was given to the animals, mixed with the same amount of water, so that the end concentration of the preservatives was actually only half of the value related to the dry substance. This way the formic acid- and sorbic acid-concentration in the wet feed was about 10 times the amount approved in the norm and that of sodium benzoate was about 5 times higher. By gradually increasing the doses, the animals got used to the preservatives. In the dosage of sodium benzoate it was found that the animals ate less of the feed that contained 3% of this substance so that they lagged in their weight increase. This concentration had therefore to be reduced. After feeding the diets for four weeks, the feed both of the test- and of the control animals was supplemented with paprika puree containing 45 mu g carotene per day an animal, while the feeding of the preservatives was continued. The manner of dosage is described in our previous study (4). After two weeks, one half and after another two weeks the other half of the animals were sacrificed by decapitation and the vitamin-A content was determined in the lever and kidney. The analysis was carried out with the method described in our previous study (2).

2. On the basis of the first test results we continued in the second test series the investigation of the unfavorable effect of formic- and sorbic acid on the carotene-metabolism. First we observed whether these substances also exert their inhibiting effect with lower concentrations, and then we investigated their effect after prolonged feeding (six weeks). This test

series was carried out with five groups of male rats containing 20 animals each. To the feed of the individual groups were added 1.2% and 2.4% formic acid and 1.0% and 2.10% sorbic acid, resp. related to the dry feed; the fifth group served as a control. As in the first test, paprika puree was added to the feed after four weeks. After 2 weeks of carotene supply, onehalf of the animals were sacrificed by decapitation and, since the variation of the vitamin-A content in the kidney had shown on regularity in the first test, we determined only the vitamin-A content in the liver. With the other half of the animals we proceeded in a similar manner after six weeks of carotene supply. In these animals the liver and intestines were also examined histologically.

3. Finally we made the carotene-balance test in order to clarify whether preservatives influence the carotene resorption. For the balance tests the animals received for four weeks 2.4% formic acid, 2.0% sorbic acid and 1.5% sodium benzoate mixed with the feed. The test was carried out in the manner described in our previous study (4) on 5 rats each, compared to 5 control animals.

Test results

The results of the first test series are compiled in table 1.

Table 1 Effect of preservatives on the vitamin-A content of the liver in rats

addition of preservative	weight of liver	vitamin A: IU per g liver	vitamin A: IU in total liver	vitamin A: IU in total kidney
	after 2 weeks carotene supply			
control animals	10.0 ± 0.2	16.6	166 ± 35 ***	26 ± 7.7
2.4% formic acid	9.8 ± 0.65	10.9	107 ± 18	18 + 1.4
2.0% sorbic acid	11.0 ± 0.18	11.7	129 ± 47	38 ±16.4
1.5% sodium benzoate	10.1 ± 0.86	16.5	167 ± 30	19 ±11.3
	after 4 weeks carotene supply			
control	10.8 ± 0.67	27.4	297 ± 64	30
2.4% formic acid	10.2 ± 1.09	25.2	261 ± 69	29
2.0% sorbic acid	10.4 ± 1.29	29.0	298 ± 69	32
1.5% sodium benzoate	10.4 ± 1.47	32.6	340 ± 78	32

*** less than 1%

The results show that, with the supply of formic and sorbic acid, the vitamin-A content of the liver was lower after two weeks than in the control - in the formic acid group this difference is significant - while in the group which received sodium benzoate, the vitamin-A content of the liver did not differ from that of the control animals. After 4 weeks the vitamin-A content of the liver showed no significant difference anymore in any group compared to the controls. Here too, we found in the lever of the formic acid group the least vitamin A and in the liver of the sodium benzoate group slightly more than in the control animals. The variation in the vitamin-A content of the kidney could not be evaluated because of the great fluctuations in the various data. At any rate it was found, however, that the variation in the vitamin-A content of the kidney does not go hand in hand with that of the liver.

The results of the second test series are compiled in table 2.

Table 2 Effect of preservatives on the vitamin-A content of the liver in rats

preservative added	weight of liver	vitamin A IU per g liver	IU in total liver
	after 2 weeks carotene supply		
control animals	8.5 ± 0.89	21.6	184 ± 25
1.2% formic acid	7.75± 0.65	18.2	141 ± 30**
2.4% formic acid	8.35 ± 0.98	15.1	126 ± 37***
1.0 % sorbic acid	9.3 ± 0.87	17.7	165 ± 34
2.0% sorbic acid	9.2 ± 0.91	15.7	144 ± 37*
	after 4 weeks carotene supply		
control animals	9.5 ± 1.14	46.7	553. ± 83
1.2% formic acid	9.2 ± 1.06	36.8	339 ± 71*
2.4% formic acid	9.3 ± 0.69	35.2	327 ± 37***
1.0% sorbic acid	9.55 ± 0.92	41.3	394 ± 97
2.0% sorbic acid	10.05 ± 1.13	42.0	422 ± 122

* less than 5%
** less than 2%
*** less than 1%

It can thus be said that formic and sorbic acid have an adverse effect even in a weaker concentration than in the first test. Formic acid was more harmful than sorbic acid because the vitamin-A content of theliver was much lower in animals which received formic acid in a concentration of 1.2 and 2.4% than in the controls. In the sorbic acid group the reduction was less; but if the feed contained 2.0% sorbic acid, the difference was still significant. After six weeks of carotene supply the difference between the vitamin-A content of the liver in the sorbic acid group and in the control group was not evailable, while the formic acid groups showed a significant reduction of the vitamin A stored in the liver at both concentrations.

The result of the carotene balance can be seen from fig. 1. Accordingly the animals which received formic- and sorbic acid excreted much more carotene with the feces after the supply of 90 mu g carotene in the form of paprika puree than the control animals. In the latter and in the animals consuming sodium benzoate there was no difference in the carotene excretion with the feces.

Fig. 1 Carotene content in the feces of the test animals on the first, second and third day after the supply of 90 mu g carotene.

Fig. 2 illustrates the weight increase of the individual test groups. The weight curves are practically identical, with the exception of the animals which had received sodium benzoate. Here a residue was found between the 20th and 30th day. During this time the animals received 3% sodium benzoate in the feed, which they no longer consumed completely. Starting from the 32nd day, when the sodium benzoate in the feed was reduced to 1.5%, the weight increase attained the level of the control animals, similar to the other two test groups.

The histological finding shows that the tested perservatives had caused no detectable harmful changes in the organs of the rats at the indicated concentration (formic acid 2.4%, sorbic acid 2.0%, sodium benzoate 1.5%) after

Fig. 2 Weight curve of the test animals

a - formic acid group; b - sorbic acid group; c - sodium benzoate group; d - control group

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Discussion of the results

The object of these tests was to study the effect of preservatives in atoxic quantities on the utilization of carotene. In the tests we used concentrations which neither jeopardized the growth of the animals nor caused histologically detectable changes in the organs of the latter.

According to literature data, 5% sorbic acid in the feed has no inhibiting effect on the growth and propagation after feeding for several months (1), even several years (6), the life expectancy is not shortened, and no histologically detectable changes are found in the organs. When the sorbic acid content in the feed was increased to 8%, Deuel (7) observed enlargement of the liver. The sorbic acid concentration of 1 and 2% used in our tests did not influence the growth of the animals and caused ho histologically detectable changes in the organs of the test animals, but a certain enlargement of the liver could be observed, compared to the control animals.

We find contradictory data about the tolerability of sodium benzoate in the literature. According to Deuel (7) this substance causes in a concentration of 4% in the feed after a 90 day supply neither changes in the growth of the rats and in the weight of liver and kidney, nor histologically detectable changes in the organs. Kieckenbush and Lang (8), however contend that a benzoic acid concentration of 1% is already close to the upper limit of tolerability. In their tests a great number of the animals died within a few weeks after the supply of 5% benzoate in the feed.

Our results verify the observations by Kieckenbusch and coworker, since we found in contrast to Deuel that rats consumed less of the food containing 3% sodium benzoate, lost weight after a few days, but showed no harmful symptoms at a concentration of 1.5%.

Woeblbier and Siebert (9) fed guinea pigs with a feed which contained 4% formic acid, and they found that formic acid of this concentration rapidly oxidizes in the animal organism. The concentration of 1.2 and 2.4% used by us proved harmless, both with regard to the growth of the animals and to histologically detectable changes in the organs.

On the basis of the vitamin-A content of the liver of rats it can be said that formic acid and sorbic acid have an adverse effect on the utilization of carotene, while sodium benzoate has no influence. Our findings show that, in a test period of four to six weeks, the vitamin A stored in the liver differs less fromthat of the control animals than after 2 weeks. At that time the difference is no longer significant in the sorbic acid group. There is a possibility that the organism gets used to the preservatives and becomes insensitive after a certain time to their harmful effect. In order to get a better idea, animal tests of long duration must be made.

With regard to the mechanism of action we found that formic acid and sorbic acid have an inhibiting effect on the resorption of carotene. Since preservatives reduce both the enzymatic activity and the lipase (1,6) it can be assumed that they indirectly influence the resorption of carotene by paralyzing the lipase activity.

On the basis of our findings it seemsthat the use of formic acid in preserved products containing carotene gives cause to concern.

Summary

1. The effect of formic acid,sorbic acid and sodium benzoate was studied in white male rats.

2. These tested preservatives did not influence the weight increase of the animals during ten weeks of feedings in the concentration used by us (2.4 and 1.2% formic acid, 2.0 and 1.0% sorbic acid, and 1.5% sodium benzoate, related to the dry feed), and the histological examination of the liver and intestines showed no pathological changes. In the sorbic acid group an enlargement of the liver was observed.

3. In the formic acid and sorbic acid group the vitamin A stored in the liver was lower after two, four and six weeks of carotene supply than in the control animals. The difference was greatest in the two-week test. Sodium benzoate did not effect the vitamin-A content.

4. The carotene-balance tests showed that formic and sorbic acid increase the excretion of carotene, and the deterioration of the utilization of carotene is probably due to this fact.

Summaries in French and English

References

(Translated by Carl Demrick Associates,Inc/IE/t)

Figure 1:

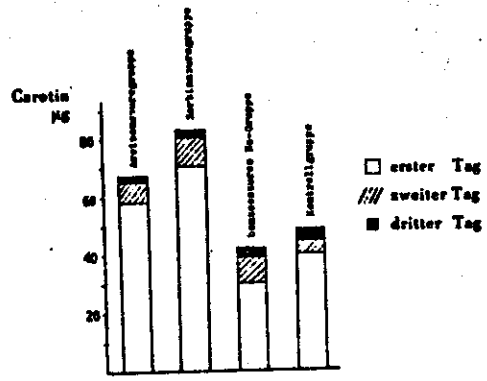

Abbildung 1. Der Carotingehalt im Faeces der Versuchstiere am ersten, zweiten und dritten Tag nach Zufuhr von 90 µg Carotin

Figure 2:

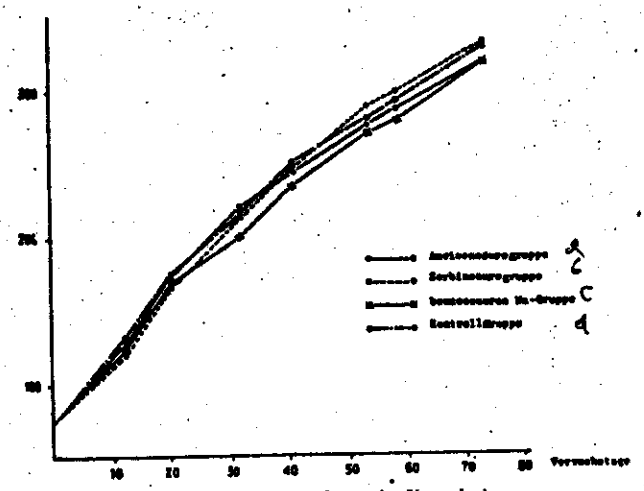

Abbildung 2. Gewichtskurve der Versuchstiere

Bibliography:

LITERATUR

1. *Souci, S. Walter* und *E. Morgenthaler:* Fremdstoffe in Lebensmitteln mit besonderer Berücksichtigung der Konservierung, München 1958. — 2. *Kramer, M.* und *R. Tarján:* Int. Z. Vitaminforsch. 1958, *28*, 289. — 3. *Kramer, M.* und *R. Tarján:* Int. Z. Vitaminforsch. 1959, *30*, 49. — 4. *Kramer, M.* und *R. Tarján:* Int. Z. Vitaminforsch. 1960, *30*, 271. — 5. *Tarján, R., E. Sándi* und *A. Dénes:* Acta phys. Acad. Sci. Hung. 1954, *5*, 313. — 6. *Lang, K:* Arzneimittelforschung 1960, *10*, 997. — 7. *Deuel, H. J., R. Alfin-Slater, C. S. Weil* und *H. F. Smith jr.:* Food Res. 1954, *19*, 1. — 8. *Kieckenbusch, W.* und *K. Lang:* Arzneimittelforschung 1960, *10*, 1001. — 9. *Wöhlbier W.* und *R. Siebert:* Tierernähr. Futtermittelk. 1940, *4*, 107.

muscle) n'a qu'un rôle fonctionnel lié à l'activité physiologique locale de l'organe.

Quoi qu'il en soit, nos résultats indiquent que, à peine a-t-elle atteint les organes, la T est rapidement phosphorylée et fixée dans les cellules, presque toujours sous forme de TDP.

En ce qui concerne, les autres composés thiaminiques, seul le contenu en T libre présente quelque intérêt, quoique son action soit limitée au foie et au rein.

Au cours de ces expériences, il n'a pas été possible de mettre en évidence, pour la teneur en TMP et en TTP, des variations particulières capables d'en éclairer l'éventuelle signification physiologique.

ZUSAMMENFASSUNG

Über den Gehalt der Rattengewebe an Thiaminphosphorsäureester nach Behandlung mit Thiamin. Von G. Rindi, L. de Giuseppe und L. De Caro.

Nach Behandlung von B_1-avitaminotischen Ratten mit Thiamin ist der Gehalt der Gewebe an Thiamin und seiner Phosphorsäureester gleich, wie nach Injektionen von Thiaminmonophosphat. Diese Tatsache spricht für eine rasche Dephosphorylierung des Thiaminmonophosphats. Der Gehalt an Thiamindiphosphat ist in den Geweben verschieden. Das Thiamin wird im Organismus rasch phosphoryliert und in den Zellen als Thiamindiphosphat fixiert. Am wichtigsten ist die Bestimmung des freien Thiamins, dessen Wirkungen sich auf Leber und Niere limitieren.

SUMMARY

The Levels of Thiamine Phosphoric Esters in Rat Tissues after Treatment with Thiamine. By G. Rindi, L. de Giuseppe and L. Caro.

Injection of thiamine has the same effect as injection of thiamine pyrophosphoric esters on the thiamine and ester contents of the tissues of rats deficient in vitamin B_1, which indicates the occurrence of a rapid dephosphorylation of thiamine monophosphate. Thiamine is rapidly phosphorylated in the body and fixed as thiamine diphosphate in the cells. The amount of thiamine diphosphate varies with the tissue. The important determination is that of free thiamine, whose action is limited to liver and kidneys.

BIBLIOGRAPHIE

1. *Rindi, G.* et *L. de Giuseppe:* Intern. Zschr. Vitaminf. 1961, *31*, 321. — 2. *De Caro, L., G. Rindi* et *L. de Giuseppe:* Intern. Zschr. Vitaminf. 1961, *31*, 333. — 3. *Rindi, G., G. Ferrari* et *V. Perri:* Intern. Zschr. Vitaminf. 1954, *25*, 210. — 4. *Rindi, G.* et *L. de Giuseppe:* Biochem. J. 1961, *78*, 602. — 5. *Westenbrink, H. G. K., E. P. Steyn-Parvé* et *J. Goudsmit:* Enzymologia 1943, *11*, 23. — 6. *van Marken Lichtenbelt* et *E. Florijn:* Acta Med. Scand. 1955, *152*, 259.

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Direktor: Dr. R. Tarján

Die Wirkung von Konservierungsmitteln auf die Verwertung von Carotin

Von Magdalene Kramer und R. Tarján

Eingegangen am 27. Nov. 1961

In den Wintermonaten wird ein gewisser Teil des Vitamin-A-Bedarfes der Bevölkerung durch den Carotingehalt einiger Gemüse- und Obstkonserven gedeckt, bei deren Herstellung normentsprechende Konservierungsmittel verwendet werden.

Bekanntlich üben Konservierungsmittel auf die Aktivität der verschiedenen Enzyme eine hemmende Wirkung aus (1) und können daher auch die einzelnen Stoffwechselvorgänge beeinflussen. Es ist deshalb anzunehmen, daß sie hinsichtlich der Carotinverwertung auch nicht indifferent sind. Gemäß der uns zur Verfügung stehenden Fachliteratur scheint man sich bis jetzt mit der Wirkung der Konservierungsmittel auf die Verwertung von Carotin nicht befaßt zu haben. Da diese Frage jedoch von praktischer Bedeutung ist, beabsichtigen wir, nach Untersuchung der verschiedenen natürlichen diätetischen Faktoren (2, 3) und der Metallverunreinigungen in den Lebensmitteln (4), den etwaigen Einfluß der in Ungarn bewilligten und von der Industrie verwendeten Konservierungsmittel auf die Verwertung von Carotin, festzustellen.

Experimenteller Teil

Zur Klärung dieser Frage führten wir folgende Tierversuche durch:

a) Bei Verabreichung von Konservierungsmittelmengen, die den Futterverbrauch und das Wachstum der Versuchstiere nicht beeinflußten und Zufuhr von bestimmten Carotingaben, wurde der Vitamin-A-Gehalt in der Leber und Niere bestimmt und mit dem Vitamin-A-Depot der Kontrolltiere verglichen.

b) Mit Konservierungsmitteln, die die Carotinverwertung beeinflussen, wurde der Versuch mit kleineren Dosen wiederholt und anschließend die Wirkung nach längerer Verfütterung beobachtet.

c) Um den Wirkungsmechanismus zu klären, wurde bei Verabreichung von verschiedenen Konservierungsmitteln das Ausmaß der Carotinausscheidung untersucht, damit die etwaige Wirkung auf die Carotinresorption und deren Ausmaß bestimmt werden kann.

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1. In der ersten Versuchsreihe wurden 40 achtwöchige weiße männliche Wistar-Ratten in vier Gruppen zu je 10 Tieren eingeteilt. Als Grundfutter erhielten die Tiere die in unseren früheren Versuchen angewendete mit Kasein ergänzte und Carotin nur in Spuren enthaltende Getreidemischung (5). Zu dem Trockenfutter der ersten Gruppe mischten wir 2,4 % Ameisensäure, zu dem der zweiten 2,0 % Sorbinsäure, zu dem der dritten 1,5 % benzoesaures Natrium. Die vierte Gruppe diente als Kontrolle. Ameisensäure und benzoesaures Natrium wurden in Form einer 10 %igen wäßrigen Lösung dem Futter beigemischt. Zur Auflösung der Sorbinsäure benutzten wir KOH in einer Menge, die die Lösung auf pH = 7 brachte. Das Futter wurde den Tieren mit gleicher Menge Wasser vermischt verabreicht, so daß die Endkonzentration der Konservierungsmittel eigentlich nur die Hälfte des auf die Trokkensubstanz berechneten Wertes betrug. Auf diese Weise war im feuchten Futter die Ameisensäure- und Sorbinsäurekonzentration etwa das 10fache der in der Norm bewilligten Menge und die des benzoesauren Natriums etwa das 5fache. Durch allmähliche Erhöhung der Gaben gewönten sich die Tiere an die Konservierungsmittel. Bei Dosierung des benzoesauren Natriums stellte es sich heraus, daß die Tiere vom Futter, das 3 % dieser Substanz enthielt, weniger verzehrten, demzufolge in ihrer Gewichtszunahme ein Rückstand eintrat. Diese Konzentration mußte daher verringert werden. Nach vierwöchiger Verfütterung der Diäten wurde das Futter sowohl der Versuchs-, wie auch der Kontrolltiere, bei weiterer Verabreichung der Konservierungsmittel, täglich je Tier mit 45 μg Carotin enthaltendem Paprikapüree ergänzt. Die Art der Dosierung ist in unserer früheren Arbeit (4) beschrieben. Nach zwei Wochen wurde die eine Hälfte, nach weiteren zwei Wochen die andere Hälfte der Tiere durch Dekapitation getötet und in Leber und Niere der Gehalt an Vitamin A bestimmt. Die Analyse führten wir mit der in unserer früheren Arbeit (2) beschriebenen Methode durch.

2. An Hand der ersten Versuchsergebnisse setzten wir in der zweiten Versuchsreihe die Untersuchung der ungünstigen Wirkung der Ameisen- und Sorbinsäure auf den Carotinstoffwechsel fort. Zuerst beobachteten wir, ob diese Substanzen ihre hemmende Wirkung auch bei schwächerer Konzentration ausüben und dann untersuchten wir ihre Wirkung nach einer längeren — sechswöchigen — Verfütterung. Diese Versuchsserie wurde mit fünf Tiergruppen zu je 20 abgesetzten männlichen Ratten durchgeführt. Dem Futter der einzelnen Gruppen wurde auf Trockenfutter berechnet, 1,2 und 2,4 % Ameisensäure, bzw. 1,0 und 2,0 % Sorbinsäure beigemischt, die fünfte Gruppe diente als Kontrolle. Wie

beim ersten Versuch wurde dem Futter nach vier Wochen Paprikapüree zugesetzt. Nach zwei Wochen Carotinzufuhr töteten wir die eine Hälfte der Tiere durch Dekapitation und nachdem im ersten Versuch die Veränderung im Vitamin-A-Gehalt der Niere keine Regelmäßigkeit aufwies, bestimmten wir lediglich den Vitamin-A-Gehalt der Leber. Mit der anderen Hälfte der Versuchstiere verfuhren wir in ähnlicher Weise nach sechswöchiger Carotinzufuhr. Bei diesen wurden Leber und Eingeweide auch histologisch untersucht.

3. Zum Schluß machten wir den Carotin-Balance-Test, um klarzustellen, ob Konservierungsmittel die Carotinresorption beeinflussen. Zur Balance-Untersuchung wurde den Tieren 4 Wochen hindurch mit 2,4 % Ameisensäure, 2,0 % Sorbinsäure, bzw. 1,5 % benzoesaurem Natrium vermischtes Futter verabreicht. Die Untersuchung wurde in der in unserer früheren Arbeit (4) beschriebenen Weise an je 5 Ratten, gegenüber 5 Kontrolltieren, durchgeführt.

Versuchsergebnisse

Die Ergebnisse der ersten Versuchsserie sind in Tabelle 1 zusammengefaßt.

Tabelle 1. Die Wirkung von Konservierungsmitteln auf den Vitamin-A-Gehalt der Leber bei Ratten

Konservierungsmittelzusatz	Gewicht der Leber	Vitamin A: IE pro g Leber	Vitamin A: IE in der Gesamtleber	Vitamin A: IE in der Gesamtniere
	nach zweiwöchiger Carotinzufuhr			
Kontrolltiere	10,0 ± 0,2	16,6	166 ± 35	26 ± 7,7
2,4 % Ameisensäure	9,8 ± 0,65	10,9	107 ± 18***	18 ± 1,4
2,0 % Sorbinsäure	11,0 ± 0,18	11,7	129 ± 47	38 ± 16,4
1,5 % benzoesaures Natrium	10,1 ± 0,86	16,5	167 ± 30	19 ± 11,3
	nach vierwöchiger Carotinzufuhr			
Kontrolltiere	10,8 ± 0,67	27,4	297 ± 64	30
2,4 % Ameisensäure	10,2 ± 1,09	25,2	261 ± 83	29
2,0 % Sorbinsäure	10,4 ± 1,29	29,0	298 ± 69	32
1,5 % benzoesaures Natrium	10,4 ± 1,47	32,6	340 ± 78	32

*** < 1 %

Die Ergebnisse zeigen, daß bei Zufuhr von Ameisen- und Sorbinsäure der Vitamin-A-Gehalt der Leber nach zwei Wochen niedriger war, als bei den Kontrollen — bei der Ameisensäuregruppe ist dieser Unterschied signifikant — hingegen unterscheidet sich bei der Gruppe, die benzoesaures Natrium erhielt, der Vitamin-A-Gehalt der Leber von dem der Kontrolltiere nicht. Nach 4 Wochen

wies, im Vergleich zu den Kontrollen, der Vitamin-A-Gehalt der Leber bei keiner Gruppe mehr einen signifikanten Unterschied auf. Auch hier fanden wir in der Leber der Ameisensäuregruppe das wenigste Vitamin A und in der Leber der benzoesauren Na-Gruppe etwas mehr, als bei den Kontrollen. Die Veränderung im Vitamin-A-Gehalt der Niere konnte infolge der großen Schwankungen in den einzelnen Daten nicht ausgewertet werden. Jedenfalls konnte man jedoch feststellen, daß die Veränderung im Vitamin-A-Gehalt der Niere mit dem in der Leber nicht parallel verläuft.

Die Ergebnisse der zweiten Versuchsreihe sind in Tabelle 2 veranschaulicht.

Tabelle 2. Die Wirkung von Konservierungsmitteln auf den Vitamin-A-Gehalt der Leber bei Ratten

Konservierungsmittelzusatz	Gewicht der Leber	Vitamin A	
		IE pro g Leber	IE in der Gesamtleber
	nach zweiwöchiger Carotinzufuhr		
Kontrolltiere	8,5 ± 0,89	21,6	184 ± 25
1,2 % Ameisensäure	7,75 ± 0,65	18,2	141 ± 30**
2,4 % Ameisensäure	8,35 ± 0,98	15,1	126 ± 37***
1,0 % Sorbinsäure	9,3 ± 0,87	17,7	165 ± 34
2,0 % Sorbinsäure	9,2 ± 0,91	15,7	144 ± 37*
	nach sechswöchiger Carotinzufuhr		
Kontrolltiere	9,5 ± 1,14	46,7	443 ± 83
1,2 % Ameisensäure	9,2 ± 1,06	36,8	339 ± 71*
2,4 % Ameisensäure	9,3 ± 0,69	35,2	327 ± 37***
1,0 % Sorbinsäure	9,55 ± 0,92	41,3	394 ± 97
2,0 % Sorbinsäure	10,05 ± 1,13	42,0	422 ± 122

* $P < 5\,\%$
** $P < 2\,\%$
*** $P < 1\,\%$

Hieraus kann festgestellt werden, daß Ameisen- und Sorbinsäure auch in einer schwächeren Konzentration, als im ersten Versuch angewendet, ungünstig wirken. Ameisensäure wirkte schädigender als Sorbinsäure, denn der Vitamin-A-Gehalt der Leber bei Tieren, die Ameisensäure in einer Konzentration von 1,2 und 2,4 % erhielten, war bedeutend niedriger als bei den Kontrollen. Bei den Sorbinsäuregruppen war die Verminderung geringer, wenn das Futter jedoch 2,0 % Sorbinsäure enthielt, war der Unterschied noch signifikant. Nach sechswöchiger Carotinzufuhr war die Differenz zwischen dem Vitamin-A-Gehalt der Leber bei der Sorbinsäure- und der Kontrollgruppe nicht bewertbar, wogegen die Ameisensäuregruppen, bei beiden Konzentrationen, eine signifikante Verminderung des in der Leber gespeicherten Vitamins A aufwiesen.

Das Ergebnis der Carotin-Balance ist aus Abb. 1 ersichtlich. Demgemäß schieden die Tiere, die Ameisen- und Sorbinsäure erhielten, nach Zufuhr von 90 µg Carotin in Form von Paprikapüree, mit den Faeces bedeutend mehr Carotin aus als die Kontrolltiere. Bei letzteren und den benzoesaures Natrium konsumierenden Tieren war in der Carotinausscheidung mit den Faeces kein Unterschied zu beobachten.

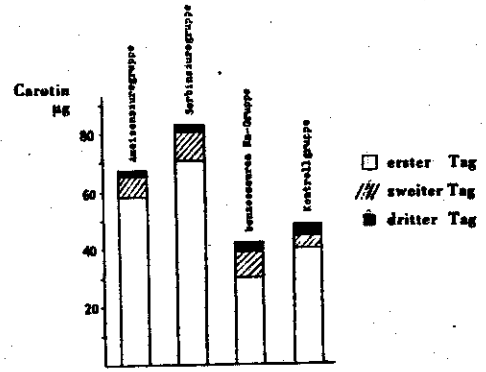

Abbildung 1. Der Carotingehalt im Faeces der Versuchstiere am ersten, zweiten und dritten Tag nach Zufuhr von 90 µg Carotin

Abb. 2 veranschaulicht die Gewichtszunahme der einzelnen Versuchsgruppen. Die Gewichtskurven sind nahezu gleich, mit Ausnahme der Tiere, die benzoesaures Natrium erhielten. Hier zeigt sich zwischen dem 20. und 30. Tag ein Rückstand. Während dieser Zeit erhielten die Tiere 3 % benzoesaures Natrium im Futter, das sie nicht restlos verzehrten. Vom 32. Tag an, als das benzoesaure Natrium im Futter auf 1,5 % herabgesetzt wurde, erreichte die Gewichtszunahme, ähnlich den anderen zwei Versuchsgruppen, das Niveau der Kontrolltiere.

Der histologische Befund zeigte, daß die untersuchten Konservierungsmittel in der angegebenen Konzentration (Ameisensäure 2,4 %, Sorbinsäure 2,0 %, benzoesaures Na 1,5 %) nach zehnwöchiger Verfütterung, in den Organen der Ratten keine nachweisbaren schädlichen Veränderungen verursacht hatten.

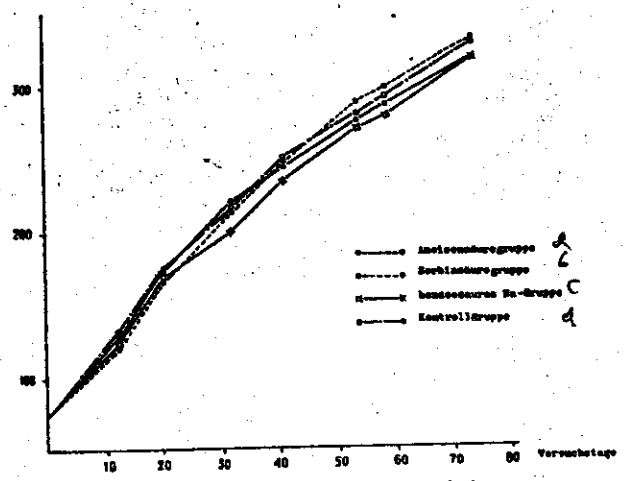

Abbildung 2. Gewichtskurve der Versuchstiere

Besprechung der Ergebnisse

Der Zweck dieser Versuche war die Wirkung von Konservierungsmitteln in atoxischen Mengen auf die Verwertung von Carotin zu untersuchen. Bei den Versuchen verwendeten wir Konzentrationen, die weder das Wachstum der Tiere gefährdeten, noch in den Organen derselben histologisch nachweisbare Veränderungen hervorriefen.

Nach Literaturangaben verursacht 5 % Sorbinsäure im Futter nach mehrmonatlicher (1), ja selbst nach mehrjähriger (6) Zufuhr, keine hemmende Wirkung auf Wachstum und Vermehrung aus, die Lebensdauer wird nicht verkürzt und sind in den Organen keine histologisch nachweisbaren Veränderungen zu beobachten. Bei Erhöhung des Sorbinsäuregehaltes im Futter auf 8 % beobachtete Deuel (7) eine Vergrößerung der Leber. Die in unserem Versuch angewendete Sorbinsäurekonzentration von 1 und 2 % beeinflußte das Wachstum der Tiere nicht und verursachte keine histologisch nachweisbaren Veränderungen in den Organen der Versuchstiere, doch konnte, den Kontrollen gegenübergestellt, eine gewisse Vergrößerung der Leber beobachtet werden.

Über die Tolerierbarkeit von benzoesaurem Natrium finden wir in der Literatur widersprechende Angaben. Nach Deuel (7) verursacht diese Substanz in einer Konzentration von 4 % im Futter,

bei 90tägiger Zufuhr, weder Veränderungen im Wachstum der Ratten und im Gewicht der Leber und Niere, noch histologisch nachweisbare Veränderungen in den Organen. Kieckenbusch und Lang (8) hingegen behaupten, daß eine Benzoesäurekonzentration von 1 % schon nahe der oberen Grenze der Ertragbarkeit liegt. Bei ihrem Versuch verendete ein großer Teil der Tiere nach Zufuhr von 5 % Benzoat im Futter binnen wenigen Wochen.

Unsere Ergebnisse bekräftigen die Beobachtungen von Kieckenbusch und Mitarbeiter, da wir im Gegensatz zu Deuel die Erfahrung machten, daß Ratten vom Futter, das 3 % benzoesaures Natrium enthält, weniger verzehren, nach wenigen Tagen an Gewicht verlieren, jedoch bei einer Konzentration von 1,5 % keine nachteiligen Symptome aufweisen.

Wöhlbier und Siebert (9) verabreichten Meerschweinchen ein Futter, das 4 % Ameisensäure enthielt und fanden, daß Ameisensäure dieser Konzentration im tierischen Organismus rasch oxydiert. Die von uns angewendete Konzentration von 1,2 und 2,4 % erwies sich sowohl auf Wachstum, als auch im Hinblick auf histologisch nachweisbare Veränderungen in den Organen als unschädlich.

Auf Grund des Vitamin-A-Gehaltes der Leber von Ratten kann festgestellt werden, daß Ameisensäure und Sorbinsäure die Carotinverwertung ungünstig beeinflussen, hingegen benzoesaures Natrium auf dieselbe keinen Einfluß ausübt. Unsere Befunde zeigen, daß bei einer Versuchsdauer von vier und sechs Wochen das in der Leber gespeicherte Vitamin A von dem der Kontrolltiere weniger abweicht als nach zwei Wochen. Zu jenem Zeitpunkt ist der Unterschied bei der Sorbinsäuregruppe nicht mehr signifikant. Die Möglichkeit besteht, daß sich der Organismus an Konservierungsmitteln gewöhnt und nach einer gewissen Zeit der schädlichen Wirkung gegenüber unempfänglich wird. Um hierüber ein klares Bild zu erhalten, müssen Tierversuche von langer Dauer durchgeführt werden.

In bezug auf den Wirkungsmechanismus stellten wir fest, daß Ameisensäure und Sorbinsäure auf die Resorption von Carotin hemmend wirken. Da Konservierungsmittel sowohl die Enzymtätigkeit als auch die Lipase (1, 6) vermindern, kann angenommen werden, daß sie durch die Lähmung der Lipaseaktivität indirekt die Carotinresorption beeinflussen.

Auf Grund unserer Ergebnisse scheint die Anwendung von Ameisensäure bei carotinhaltigen konservierten Erzeugnissen besorgniserregend.

Zusammenfassung

1. Bei weißen männlichen Ratten wurde die Wirkung von Ameisensäure, Sorbinsäure und benzoesaurem Natrium auf die Carotinverwertung untersucht.

2. Die untersuchten Konservierungsmittel beeinflußten während zehnwöchiger Verfütterung in der von uns angewendeten Konzentration (2,4 und 1,2 % Ameisensäure, 2,0 und 1,0 % Sorbinsäure, 1,5 % benzoesaures Natrium auf Trockenfutter berechnet) die Gewichtszunahme der Versuchstiere nicht und zeigte die histologische Untersuchung der Leber und Eingeweide keine pathologischen Veränderungen. Bei der Sorbinsäuregruppe war eine Vergrößerung der Leber zu beobachten.

3. Bei den Ameisensäure- und Sorbinsäuregruppen war das in der Leber gespeicherte Vitamin A nach zwei-, vier- und sechswöchiger Carotinzufuhr geringer als bei den Kontrolltieren. Der Unterschied war im Zweiwochenversuch der bedeutendste. Benzoesaures Natrium beeinflußte den Vitamin-A-Gehalt der Leber nicht.

4. Die Carotin-Balance-Untersuchungen ergaben, daß Ameisen- und Sorbinsäure die Carotinausscheidung erhöhen und die Verschlechterung der Carotinverwertung vermutlich diesem Umstand zuzuschreiben ist.

RESUME

Effet de produits de conservation sur l'assimilation du carotène. Par M. Kramer et R. Tarjan.

Les produits de conservation (acide formique à 2,4 ou 1,2 %) n'exercent aucune influence sur l'augmentation de poids des rats utilisés pour les expériences. L'examen histologique du foie et des viscères ne révèle aucune modification pathologique. Seul le foie du groupe traité à l'acide sorbique était agrandi. Après l'administration de carotène, le taux de vitamine A était plus faible dans le foie des rats traités à l'acide formique ou à l'acide sorbique que dans celui des sujets témoins. La plus profonde différence se manifeste lors des essais durant deux semaines. Le benzoate de soude n'exerce aucune influence sur le taux de vitamine A dans le foie. L'acide formique et l'acide sorbique accroissent l'élimination du carotène; ainsi s'explique peut-être l'utilisation moindre du carotène.

SUMMARY

Effect of Preservatives on the Utilisation of Carotene. By M. Kramer and R. Tarjan.

The preservatives formic acid (2.4 or 1.3 %), sorbic acid (2.0 or 1.0 %) and sodium benzoate (1.5 %) have no effect on the increase in weight of experimental rats nor do they produce evidence of any histopathological changes in liver and intestinal tract; sorbic acid, however, caused liver enlargement. After administration of carotene, the vitamin A in the livers of animals of the formic and sorbic acid groups was lower than that of control animals; the difference was greatest in a 2-week experiment. Benzoic acid had no effect on the liver vitamin A content. Both formic and sorbic acid raise the level of carotene excretion, which perhaps explains the reduced carotene utilisation.

LITERATUR

1. *Souci, S. Walter* und *E. Morgenthaler:* Fremdstoffe in Lebensmitteln mit besonderer Berücksichtigung der Konservierung, München 1958. — 2. *Kramer, M.* und *R. Tarján:* Int. Z. Vitaminforsch. 1958, *28*, 289. — 3. *Kramer, M.* und *R. Tarján:* Int. Z. Vitaminforsch. 1959, *30*, 49. — 4. *Kramer, M.* und *R. Tarján:* Int. Z. Vitaminforsch. 1960, *30*, 271. — 5. *Tarján, R., E. Sándi* und *A. Dénes:* Acta phys. Acad. Sci. Hung. 1954, *5*, 313. — 6. *Lang, K:* Arzneimittelforschung 1960, *10*, 997. — 7. *Deuel, H. J., R. Alfin-Slater, C. S. Weil* und *H. P. Smith jr.:* Food Res. 1954, *19*, 1. — 8. *Kieckenbusch, W.* und *K. Lang:* Arzneimittelforschung 1960, *10*, 1001. — 9. *Wöhlbier W.* und *R. Siebert:* Tierernähr. Futtermittelk. 1940, *4*, 107.

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Untersuchungen über den Vitamin-Stoffwechsel bei Geflügel

I. Mitteilung:

Der Vitamin-A-Gehalt im Serum und in der Leber in Abhängigkeit vom Alter bei Hähnen

Von Walter Feldheim*, Mikuláš Gažo und Olga Sladka

Eingegangen am 25. Nov. 1961

Bei der Intensivhaltung von Geflügel werden mit dem Futter Vitamin-A-Dosen an die Tiere verabfolgt, die das 2—3fache des physiologischen Bedarfs decken könnten. Die über den normalen Bedarf hinausgehende Menge soll prophylaktisch den bei Infektionen oder Stress eintretenden Mehrverbrauch decken. Untersuchungen über die Vitamin-A-Speicherung bei hohem Vitamin-A-Gehalt des Futters wurden bereits durchgeführt. Dagegen fehlen noch Angaben über den Verlauf der Vitamin-A-Gehalte in Leber und Serum, wenn den Tieren nur eine suboptimale Vitamin-A-Menge zur Verfügung steht. In den hier vorliegenden Untersuchungen wurde eine Standardfuttermischung mit einem Vitamin-A-Gehalt von 2000 IE/kg an New-Hampshire-Hähnchen verfüttert. Die Versuche wurden parallel in Ivanka und Rehbrücke durch-

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Adaptation of Some Digestive Glands to the Introduction of Chemical Food Preservatives (Sorbic Acid and Potassium-Sorbate)

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With the development of chemistry, in many countries of the world, various chemical food preservatives are being employed for the purpose of preventing the spoilage of food products. Among them, in the manufacture of various preserves (canned goods), and in the confectionery industry, use is made of sorbic acid and of its salt -- potassium-sorbate. As an antiseptic, this preservative acts, in equal measure, on yeast and on mold microorganisms.

Melnick believes that the action of sorbic acid is based on inhibiting the activity of the dehydrogenase system, which plays an important part in microorganisms. Whitaker likewise has observed the inhibition of some sulfhydryl enzymes.

Along with its excellent preserving property, attention is called to the fact that sorbic acid, repres nting a homologous compound of fatty acids, on finding its way into the organism, just as these acids, participates in an intermediate metabolism. Deuel, and his coworkers, has indicated that in starvation, sorbic acid converts to ketone bodies in the same manner, as, for example, caproic acid. Under normal feeding conditions, sorbic acid, in the organism, decomposes into carbonic acid (carbon dioxide) and water. Acute, subacute and chronic experiments were conducted by Deuel and Smyth, and at the present time, the problem is under study by A. D. Ignat'ev.

On the basis of experiments that have been carried out on animals, it has turned out to be that an LD_{50} of sorbic acid and its salt is quite high, and on conversion to 1 kilogram of body weight, it will fluctuate between 5 and 10 grams. In subacute and chronic experiments with sorbic acid, which has been added to feed in an amount of less than 8%, no pathological deviations were disclosed.

Thus, the impression takes shape that sorbic acid possesses favorable hy-

gienic properties.

In the present paper we have made an attempt, by employing such highly sensitive tests as the exacrinous activity of the liver and of the pancreas, to clarify in what manner sorbic acid exerts an influence on juice formation and on the composition of digestive juices, and whether one could count on the development of an adaptation of the organism in the case of a prolonged use of the abovementioned preservatives.

Procedure

In the experiment, there were employed 108 white female rats with a weight of 200 - 300 grams. In an acute experiment, there was administered a 1/5 part of the above-indicated LD_{50}, i.e., 0.2 grams of sorbic acid and potassium-sorbate, calculated according to the amount of sorbic acid. Preservatives were added to the feed received once by the animals.

Subacute experiments were carried out over a period of 3 months. At the same time, there were added to the feed sorbic acid and potassium-sorbate in an amount that was equal to 2 and 0.25%. The control animals received the same amount of feed without any additions.

Following a one-time and 3-month feeding, an operation was carried out with the removal of a fistula of the bile and pancreatic ducts. Preliminarily, the rats were anesthetized intraperitoneally by means of administering urethane, figuring on 0.1 grams for 100 grams of body weight. From a polyethylene tube, which had been introduced underneath the liver in the bile duct, bile was collected; from a tube that had been introduced into the duct in front of the duodenum, with a constant cooling, pancreatic juice was collected over a period of 2 - 3 hours. A measurement was made of the amount of bile and pancreatic juice, and after that, a determination was made in the file of: the overall amount of albumin, cholesterol, bilirubin, and the content of sodium and potassium. In the pancreatic juice, a determination was made of the overall amount of albumin, and of the activity of trypsin, chymotrypsin, lipase (the

methods of Schon and of his coworkers), and of amylase in accordance with the method of Smyth and Roe. All the data were calculated for a time period of 60 minutes.

Results

In acute experiments there were observed considerable changes in the bile indices under study.

Upon administering both sorbic acid, as well as potassium-sorbate, the amount of bile diminished. There was a reduction in the overall amount of albumin and in the sodium content. On the contrary, the amount of bilirubin and cholesterol in the bile, under the influence of potassium-sorbate, increased. The potassium content in the bile did not change.

In a subacute experiment, on feeding preservatives in doses equal to 2 and 0.25%, our attention is called to the circumstance that, following a 3-month feeding of the animals, with the addition of preservatives, not in a single dosage were there observed any changes either in the amountof bile being secreted, or in the overall amount of albumin, or in the content of potassium and sodium in it. In the content of cholesterol and bilirubin, there was observed a certain tendency towards an increase, but the deviations in comparison with the control experiments, were much smaller and were not as unambiguous as in the acute experiment.

Under the influence of sorbic acid, the amount of pancreatic juice, the overall albumin content, and the activity of the lipase and of the amylase increased. Upon the administration of potassium-sorbate, the activity of chymotrypsin diminished.

In a subacute experiment, under the influence of 2% preservatives, there was a diminution in the amountof pancreatic juice, in the overall content of albumin, and in the activity of the chymotrypsin and amylase. The activity of the lipase diminished only under the influence of sorbic acid. This picture is opposte to the one that was observed in the acute experiment, where these indices rather increased.

In the subacute experiment, upon employing 0.25% preservatives, results were obtained that practically coincided with the data of the acute experiment. In comparison with the control animals, there was observed, here, an increase in the amount of pancreatic juice being secreted, in the overall albumin content in it, and also, in the activity of all the enzymes.

Discussion of the Results

The results of the studies have shown that under the influence of serbic acid and potassium-sorbate, the directivity of changes in the exacrinous activity of the liver and pancreas, in acute experiments, is a varying one. Whereas a one-time administered dose of the preservative (0.2 grams) diminishes the secretion of bile and the amount of the substances that enter into its composition, the secretion of pancreatic juice rather increases. In the event of a single, high dosage of the preservative, all the indices of bile secretion, with the exception of the secretion of bilirubin, have a tendency towards reduction; this makes it possible to express an assumption with regard to the impairment of liver function, in view of the fact that the utilization of preservatives brought about the formation of a smaller amount and of a less concentrated bile.

Our attention is particularly called to the considerable reduction in the sodium content in the bile. Gallagher andhis coworkers, also Thiers, and afterwards Varga, studied the changes in the cations in the liver under the influence of various substances that harm the liver. The latter author has noted that a subcutaneous administration of carbon tetrachloride (0.1 milliliters for 100 grams of body weight), in 8 hours, increases, by approximately twice, the level of sodium in the liver. In view of the fact that at the present time, a change in the level of liver cations, and particularly, an increase in the sodium content, is considered to be one of the sensitive symptoms of liver damage, it is assumed that a reduction in the secretion of sodium, which has its onset under the influence of sorbic acid and potassium-sorbate, represents a result of an impairment in the function of the liver tissue. For the purpose of confirming this assumption, it is necessary to study

the amount of cations in the liver and in the blood.

In a subacute experiment, with both preservative dosages, i.e., with 2 and 0.25%, given over a period of 3 months, there takes place an adaptation ofthe bile flow and albumin secretion and of salts by the liver. An increase in the content of bilirubin and cholesterol, under the influence of 2% preservatives, was much less than in the acute experiment. Upon introducing 0.25% preservatives, no deviations were disclosed, in general, in comparison with the control animals. These data attest to the adaptation of the exacrinous function of the liver, and point out that the addition to the feed of 2% preservatives does not impair the bile-formation of the liver. The data that have been obtained coincide with the observations of A. D. Ignat'ev, who has reported that in rats that had received sorbic acid, in the case of loading with carbon tetrachloride, in comparison with the control group, the detoxication capacity not only did not diminish, but rather increased

No impairments were observed in the exacrinous activity of the pancreas under the influence of preserving substances in the acute experiment. In this case, the results of the experiments once again confirmed the fact, which is well known in the literature, as to the adaptation of the composition of digestive juices to the quality of the products being used. Sorbic acid, in the same manner as the introduction of increased amounts of fatty acids, increased juice formation, and also, the activity of the lipase.

The data from the subacute experiment attest to the fact that a 2% dosage of sorbic acid, and also, of potassium-sorbate, diminished the exacrinous activity of the pancreas, whereas in the literature, the indicated dosage is evaluated as being inoperative. In histological investigations, which had been carried out following 3 months of the experiment, no pathological changes were observed.

Chronic experiments in our investigations were not carried out.

With a 0.25% dosage of preserving substances (preservatives) no diminution

in the function of the pancreas was disclosed. In the given case, there was observed, rather, some increase in the activity of the enzymes.

BIBLIOGRAPHY

Melnick, D., Food Res., 1954, 19, 44.

Whitaker, J. R., Food Res., 1959, 24, 37.

Deuel, H. J. et al., Food Res., 1954, 19, 13.

Deuel, H. J. et al., Food Res., 1954, 19, 1.

Smyth, H. T., J. Ind. Hyg. Toxicol., 1948, 30, 63.

Ignat'ev, A. D., Vopr. pitaniya (Nutritional Problems), 1965, 24, 61.

Jendrassik, L. et al., Biochem. J., 1938, 297, 81.

Schon, H. et al., Klin, Wschr., 1961, 39, 217.

Smidth, W., Roe, J. H., J. Biol. Chem., 1949, 17, 53.

Gallagher, C. H. et al., J. Path. Bact., 1956, 72, 247.

Thiers, R. E. et al., J. Biol. Chem., 1960, 235, 2130.

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АДАПТАЦИЯ НЕКОТОРЫХ ПИЩЕВАРИТЕЛЬНЫХ ЖЕЛЕЗ К ВВЕДЕНИЮ ХИМИЧЕСКИХ ПИЩЕВЫХ КОНСЕРВАНТОВ (СОРБИНОВОЙ КИСЛОТЫ И КАЛИЯ-СОРБАТА)

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С развитием химии во многих странах мира для предохранения от порчи пищевых продуктов применяют различные химические пищевые консерванты. В числе их при производстве различных консервов, в кондитерской промышленности используется сорбиновая кислота и ее соль — калий-сорбат. Как антисептик этот консервант в одинаковой мере действует на дрожжевые и плесневые микроорганизмы.

Melnick считает, что действие сорбиновой кислоты основывается на торможении активности дегидрогеназной системы, которая у микроорганизмов играет большую роль. Whitaker отмечает также торможение некоторых сульфгидрильных энзимов.

Наряду с прекрасным консервирующим свойством обращает на себя внимание то, что сорбиновая кислота, являясь гомологом жирных кислот и попадая в организм, как и эти кислоты, участвует в промежуточном обмене веществ. Deuel и сотрудники указывают на то, что при голодании сорбиновая кислота превращается в кетоновые тела, так же, как, например, капроновая кислота. При нормальных условиях питания сорбиновая кислота в организме распадается на углекислоту и воду. Острые, подострые и хронические опыты проводились Deuel и Smyth, в настоящее время вопрос изучается А. Д. Игнатьевым.

На основании опытов, проведенных на животных, оказалось, что LD_{50} сорбиновой кислоты и ее соли довольно высока и в расчете на 1 кг веса тела колеблется между 5 и 10 г. В подострых и хронических опытах с сорбиновой кислотой, добавляемой к корму в количестве менее 8%, не было обнаружено никаких патологических отклонений.

Таким образом, складывается впечатление, что сорбиновая кислота обладает благоприятными гигиеническими свойствами.

В настоящей работе мы пытались, используя такие высокочувствительные тесты, как внешнесекреторная деятельность печени и панкреатической железы, выяснить, каким образом влияет сорбиновая кислота на сокообразование и состав пищеварительных соков, и можно ли рассчитывать на возникновение адаптации организма при длительном потреблении упомянутых выше консервантов.

Методика

В эксперименте было использовано 108 белых крыс-самок весом 200—300 г. В остром опыте вводили $^1/_5$ часть указанной выше LD_{50}, т. е. 0,2 г сорбиновой кислоты и калия-сорбата, рассчитанного по количеству сорбиновой кислоты. Консерванты добавляли к корму, получаемому животными однократно.

Подострые опыты проводили в течение 3 месяцев. При этом к корму добавляли сорбиновую кислоту и калий-сорбат в количестве, равном 2 и 0,25%. Контрольные животные получали такое же количество корма без добавок.

После одноразового и 3-месячного кормления проводили операцию с выведением фистулы желчного и панкреатического протоков. Предварительно крыс наркотизировали интраперитонеально введением уретана в расчете 0,1 г на 100 г веса тела. Из полиэтиленовой трубки, введенной под печень в желчный проток, собирали желчь, из трубки, введенной в проток перед двенадцатиперстной кишкой, при постоянном охлаждении собирали панкреатический сок в течение 2—3 часов. Измеряли количество желчи и панкреатического сока и затем определяли в желчи: общее количество белка, холестерин, билирубин и содержание натрия и калия. В панкреатическом соке определяли общее количество белка, активность трипсина, химотрипсина, липазы (методы Schön и сотрудников) и амилазы по методу Smyth и Roe. Все данные рассчитывали на время — 60 минут.

Результаты

В острых опытах наблюдались значительные изменения исследуемых показателей желчи.

При введении как сорбиновой кислоты, так и калия-сорбата количество желчи уменьшалось. Снижались общее количество белка и содержание натрия. Напротив, количество билирубина и холестерина в желчи под влиянием калия-сорбата повышалось. Содержание калия в желчи не изменялось.

В подостром эксперименте при скармливании консервантов в дозах, равных 2 и 0,25%, обращает на себя внимание то обстоятельство, что после 3-месячного кормления животных с добавлением консервантов ни при одной дозировке не обнаружено изменений ни в количестве выделяемой желчи, ни в общем количестве белка, ни в содержании калия и натрия в ней. В содержании холестерина и билирубина наблюдалась некоторая тенденция к повышению, но отклонения по сравнению с контрольными опытами были гораздо меньшими и не так однозначны, как в остром опыте.

Под влиянием сорбиновой кислоты количество панкреатического сока, общее содержание белка, активность липазы

и амилазы повышались. При введении калия-сорбата активность химотрипсина уменьшалась.

В подостром опыте под влиянием 2% консервантов уменьшались количество панкреатического сока, общее содержание белка, активность химотрипсина и амилазы. Активность липазы уменьшалась только под влиянием сорбиновой кислоты. Эта картина противоположна той, которая наблюдалась в остром опыте, где эти показатели скорее повышались.

В подостром опыте при применении 0,25% консервантов получены результаты, почти совпадающие с данными острого опыта. По сравнению с контрольными животными здесь наблюдалось повышение количества выделенного панкреатического сока, общего содержания белка в нем, а также активности всех энзимов.

Обсуждение результатов

Результаты исследований показали, что под воздействием сорбиновой кислоты и калия-сорбата направленность изменений внешнесекреторной деятельности печени и панкреатической железы в острых опытах различная. В то время как однократно данная доза консерванта (0,2 г) уменьшает секрецию желчи и количество веществ, входящих в ее состав, секреция панкреатического сока скорее увеличивается. В случае однократной высокой дозировки консерванта все показатели секреции желчи, за исключением секреции билирубина, имеют тенденцию к снижению, что позволяет высказать предположение о нарушении функции печени, так как использование консервантов вызывало образование меньшего количества и менее концентрированной желчи.

Особенно обращает на себя внимание значительное понижение содержания натрия в желчи. Gallagher и сотрудники, а также Thiers, затем Varga изучали изменения катионов в печени под влиянием различных веществ, повреждающих печень. Последний автор отмечает, что подкожное введение четыреххлористого углерода (0,1 мл на 100 г веса тела) через 8 часов приблизительно вдвое повышает уровень натрия в печени. Ввиду того что в настоящее время изменение уровня катионов печени и особенно повышение содержания натрия считается одним из чувствительных признаков повреждения печени, предполагается, что понижение секреции натрия, наступающее под влиянием сорбиновой кислоты и калия-сорбата, является следствием нарушения функции печеночной ткани. Для подтверждения этого предположения необходимо исследование количества катионов в печени и крови.

В подостром опыте при обеих дозировках консервантов, т. е. при 2 и 0,25%, даваемых в течение 3 месяцев, имеет мес-

то адаптация желчеотделения и выделения белка и солей печенью. Повышение содержания билирубина и холестерина под влиянием 2% консервантов было гораздо меньшим, чем в остром опыте. При введении 0,25% консервантов никаких отклонений по сравнению с контрольными животными вообще не обнаружено. Эти данные свидетельствуют о приспособлении внешнесекреторной функции печени и говорят о том, что добавление к корму 2% консерванта не нарушает желчеобразование печени. Полученные данные совпадают с наблюдениями А. Д. Игнатьева, который сообщил, что у крыс, получавших сорбиновую кислоту в случае нагрузки четыреххлористым углеродом, по сравнению с контрольной группой детоксикационная способность не только не уменьшалась, а скорее повышалась.

Во внешнесекреторной деятельности панкреатической железы под влиянием консервирующих веществ в остром опыте нарушений не наблюдалось. В этом случае результаты опытов вновь подтвердили широко известный в литературе факт о приспособлении состава пищеварительных соков к качеству потребляемых продуктов. Сорбиновая кислота, так же как и введение повышенных количеств жирных кислот, увеличивала сокообразование, а также активность липазы.

Данные подострого опыта свидетельствуют о том, что 2% дозировка сорбиновой кислоты, а также калия-сорбата уменьшала внешнесекреторную деятельность панкреатической железы в то время как в литературе указанная дозировка оценивается как недействующая. При гистологических исследованиях, проведенных после 3 месяцев эксперимента, никаких патологических изменений не обнаружено.

Хронические опыты в наших исследованиях не проводились.

При 0,25% дозировке консервирующих веществ никакого уменьшения функции панкреатической железы не обнаружено. В данном случае наблюдалось скорее некоторое увеличение активности энзимов.

ЛИТЕРАТУРА

Melnick D. Food Res., 1954, 19, 44.
Whitaker J. R. Food Res., 1959, 24, 37.
Deuel H. J. et al. Food. Res., 1954, 19, 13.
Deuel H. J. J. et al. Food Res., 1954, 19, 1.
Smyth H. T. J. Ind. Hyg. Toxicol., 1948, 30, 63.
Игнатьев А. Д. Вопр. питания, 1965, 24, 61.
Jendrassik L. et al. Biochem. J., 1938, 297, 81.
Schön H. et al. Klin. Wschr., 1961, 39, 217.
Smidth W., Roe J. H. J. Biol. Chem., 1949, 17, 53.
Galiagher C. H. et al. J. Path. Bact., 1956, 72, 247.
Thiers R. E. et al. J. Biol. Chem., 1960, 235, 2130.

АДАПТАЦИЯ ОБМЕНА ТИАМИНА К ЧАСТОТЕ ПРИЕМОВ ПИЩИ

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В течение последних лет в лабораториях Tepperman и Cohn в Америке и Fábry в Праге было показано, что ограничение приема пищи кратковременными приемами с продолжительными промежутками между ними, как, например, 2 часа в день, через день или 2 раза в неделю, приводит у крыс в сравнительно короткий срок к ряду таких адаптивных изменений, как гипертрофия желудка и кишечника (Holeckova и Fábry; Fábry и Kujalová) увеличенное всасывание глюкозы и жира (Kujalová, Fábry), повышенный синтез гликогена в печени и мышцах (Fábry, Petrásek и др., 1962), увеличенная утилизация глюкозы (Hollifield, Parkson; Tepperman J. и Tepperman H.) и значительное увеличение превращения углеводов в жиры с активацией пентозо-фосфатного цикла (Tepperman J. и Tepperman H., Fábry, Petrásek, Braun, Bednárek, Horáková).

Поскольку тиамин в форме кокарбоксилазы существенно связан с углеводным обменом и транскетолаза участвует в пентозо-фосфатном цикле, возникает вопрос, не зависит ли потребность организма в тиамине и от частоты приема пищи.

Для выяснения этого вопроса мы исследовали у крыс общее содержание тиамина, содержание радиоактивного тиамина и специфическую радиоактивность тиамина в печени, а также активность транскетолазы в эритроцитах при оптимальной, субоптимальной и недостаточной обеспеченности организма тиамином в зависимости от частоты приема пищи. Крыс кормили или ad libitum или 2 часа в день.

Результаты исследований показали, что содержание общего тиамина в печени крыс соответствует содержанию тиамина в пище, т. е. оно наибольшее при оптимальном и наименьшее при субоптимальном и недостаточном обеспечении тиамином. При субоптимальном и недостаточном обеспечении тиамином нет достоверной разницы в содержании тиамина между крысами, которых кормили ad libitum или 2 часа в день. При оптимальном же обеспечении тиамином содержание его в печени адаптированных крыс ниже, чем у контрольных.

Как следовало ожидать, активность транскетолазы в эритроцитах при субоптимальном и недостаточном обеспечении витамином B_1 ниже, чем при оптимальном. Между крысами, которых кормили ad libitum или 2 часа в день, при субоптимальном и недостаточном обеспечении тиамином не наблюдается достоверной разницы, в то время как у оптимально

The Effect of Preservatives on the External Secretion of the Pancreas

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Kiserletes Orvostudomany 20, 624-633 (1968)

Among the foreign substances entering the body with food stuffs, an important role is played by preservatives, which are used widely both in industry and in households. They widespread use is particularly important in relations to diets, the feeding of patients and because of the difficulty in evaluating them with respect to their suitability for use in dietetic products. The use of the preservatives by the food industry is regulated on the basis of a nutritional health evaluation. however, such evaluations differ from country to country. The principal cause of differing opinions is that the data available for the judging of preservatives are of different origins: a minor part originates in observations of humanes, while most of them are based on animal experiments. Methods used in animal experimentation generally differ and therefore the results are frequently also different. For this reason, there is need for the application of a generally useful animal experimental method which would yield information concerning the harmful or harmless effects of different preservatives in a relatively short period of time.

Based on the above, the authors investigated the external secretional activity of pancreas and its variation with respect to the effect of different preservatives; this activity reacts sensitively to various physiological effects. The objective of the experiments thus was dual: first, to evaluate the suitability of the pancreas body for the purpose and secondly, to determine the effect of different preservatives on the pancreas function.

The following preservatives were examined:

1. The pyrocarbonic acid diethyl ester (Baycovin) proposed for introduction by the food industry is suitable mainly for the preservation of fruit juices and wines. The material has a pungent odor and irritates mucous membranes; it dissociates in storage into ethanol and CO_2. The rate of decomposition decreases with increasing ethanol and CO_2 concentrations and it is also a

function of temperature and pH.

For this reason, the time ellapsed between the application and the consumption of the material is highly important.

The oral LD_{50} of the preservative for rats is 1.57-1.39 g/kg of body weight /1/, according to Hecht /2/ it is 1.1 ml. In the experiment of the latter author, amounts of 1.5-2.5 ml/kg caused death in 12 hours preceded by symptoms of paralysis and inhibited respiration. In subacute experiments, administration of ¼ of LD_{50} i.e. 0.25 ml/kg once a week, for 4 months, caused loss of food intake and of body weight. Bornmann /1/ reported the administration of 2% fresh pyrocarbonic-diethyl ester for 30 days caused similar symptoms, however, doses of 0.5% caused no harmful effects.

2. Benzoic acid, particularly its sodium salt, is widely used in the food industry for the preservation of fruit, vegetables and certain confectionary products. Benzoic acid (orthooxysalicylic acid) can be present in the body not only as the result of outside addition, it can also be formed physiologically during intermediate metabolism, where it is attached to glycocoll and glucuronic acid and is eliminated in the form of hippuric acid in urine.

Toxicity investigations showed that the oral LD_{50} for rats is around 2-3 g/kg /3/. Chronic consumption by humans in doses of 0.5 and 1 g/day did not cause symptoms /4/ and even 12 g/day had no effect. /5/. According to animal experiments, the feeding of 2% sodium benzoate for 3 months did not cause pathological changes even with a supply of 35% casein. /6/, with feed containing 20% casein, however, it strongly interfered with reproduction and caused loss of animals /7/. The administration of 1-4% sodium benzoate with a 20% casein diet for 90 days did not cause death /8/. Land /9/ determined the critical limit between 1-2%. The use of sodium benzoate as a preservative in this country is permitted to a maximum of 0.15%.

3. The use of salicylic acid and of its sodium salt in the food industry has been prohibited in numerous countries, including our own, but a majority of households is still using it as a preservative. In addition to its well-

known pharmocological effects, irritation on the mucous membranes of the stomach and inhibition of pepsin activity. LD_{50} for rats is 1.6 g/kg per os /10/.

4. Sorbic acid and its salts are used preferentially as preservatives by the food industry, in a maximum concentration of 0.1%. In addition to its excellent preservative effect, it has the advantage that it does not interfere with the tests of the finished product, because as a 5 carbon unsaturated fatty acid, it is matabolized in the body in a manner similar to the fatty acids of food and is finally decomposed into CO_2 and water /11/. Its toxicity has been investigated -- among others -- by Deuel /12/, Ignatyev /13/, Cremer /14/, Land /15/ and Smith /16/. The oral LD_{50} of rats is 5-10 g/kg. During starvation, the value of LD_{50} is decreasing. In subacute and chronic toxicity experiments, feeding the substance in amounts of less than 5-8% with respect to dry substance did not cause pathological changes, according to results obtained by conventional methods. Sorbic acid, when fed in amounts of 0.1, 0.5 and 5% for 100 days did not change the rate of reproduction or death /17/. Under the effect of 8% sorbic acid with respect to dry matter, a slight increase in the weight of the liver was observed /12/.

Methods:

A total of 272 rats, raised in the authors laboratory (60 control, 32 pyrocarbonicethyl ester, 38 sodium benzoate, 43 sodium salicylate, 108 sorbic acid and potassium sorbate) were used in the experiments. In the experiments to determine acute effects, approx. 1/5 of the LD_{50} shown by the literature were administered to the rats, once, by a stomach syringe: i.e. 0.32 g/kg of pyrocarbonic acid diethyl ester, both freshly dissolved and thus undecomposed and after storing for 48 hours at room temperature, 0.6 g/kg of sodium benzoate, 0.32 g/kg sodium salicylate, 2 g/kg sorbic and acid and potassium sorbate calculated as sorbic acid. The controls received the same amounts of the solvent, i.e. distilled water, again through a stomach syringe. Changes occurring in response to the prolonged administration of sodium benzoate,

sodium salicylate and sorbic acid, also potassium sorbate in subacute doses, were also investigated. To the normal feed (18% protein) 2% sodium benzoate and sodium salicylate; of the sorbic acid and potassium sorbate 0.25% and 2% were mixed to the feed; this was given to rats for 4 weeks. The effect of sorbic acid was also examined in chronic experiments, by feeding it in concentrations of 0.5% and 1.5% for 22 weeks.

Following the completion of both the subacute and acute administration, by way of an acute pancreas fistula operation /18/ for 2½ hours (without the administration of secretin and carbamylcholin), the pancreas secretion was collected under cooling with ice. After determining its weight, the total protein content of the pancreas secretion /19/ and its trypsin, chimotrypsin, lipase /20/ and amylase activities were measured. The activity of trypsin is expressed in beta-naphtylamine cleaved from the substrate, the activities of chimotrypsin and lipase in betanaphtol, the activity of amylase in starch pg. The amounts are calculated for pancreas secretion volumes secreted in 60 minutes. Deviations form the control group were evaluated by the Student T test, or, if possible by the DD test. During the chronic test, the weight of the animals was observed, on some of the rats liver function /22/ tests were performed and the activities of serum lipase and chimotrypsin were determined. Finally, after the bleeding of the rats, their organs were weighed and histologically processed.

Results:

It is seen on Fig. 1 that pyrocarbonic acid diethylester in the undissociated state reduces all of the parameters measured; the change is most significant in the activities of trypsin, chimotrypsin and lipase. The same substance stored for 48 hours did not reduce the external secretion of the pancreas, but reinforced it.

Fig. 2 illustrates the situation caused by sodium benzoate. As is seen, reduction occurred in the activity of chimotrypsin only, while in the acture experiment, the lipase activity increased, however, due to the scatter the in-

crease is not significant. It is seen in Fig. 3 that under the acture effect of sodium salicylate the amount of pancreas secretion and the activity of trypsin and lipase show declining trends. Under the effect of subacute dosage, decreases were observed in each case, except the lipase activity. Pronounced declines were observed in the case of total protein content and the activity of chimotrypsin. Fig. 4 presents the results obtained with a single (acute experiment administration of sorbic acid and potassium sorbate. It is seen that compared with the control, slight decreases are found in the total protein content and in the activity of chimotrypsin activity. The activities of lipase and amylase activity rose in both cases (Due to the great scatter, the deivation is significant at the .01 level only) (Fig.4).

Figure 1 Effect of pyrocarbonic Acid diethylester of the External Secretion Activity of the Pancreas (Acute Experiment)

Key: 1. Control 2. Fresh pyrocarbonic acid diethyl ester 3. Ester stored for 48 hours 4. Amount of liquid 5. Total protein 6. Trypsin 7. Chimotrypsin 8. Lipase 9. Activity 10. amylase

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Figure 2 The Effect of Sodium Benzoate on the External Secretion Activity of the Pancreas

Key: 1. Acute experiment 2. Subacute experiment 3. Amount of liquid 4. Total protein 5. trypsin 6. chimotrypsin 7. lipase 8. amylase

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Figure 3 Effect of Sodium Salicylate on the External Secretion of the Pancreas

Key as above

Figure 4 Effect of Sorbic Acid and Potassium Sorbate on the External Secretion of the Pancreas (Acute Experiment)

Key 1. Sorbic acid 2. Potassium sorbate

Figure 5 Effect of 0.25% Sorbic Acid and Potassium Sorbate on the External Secretion of the Pancreas (Subacute Experiment)

Key as above

Figure 6 Effect of 2% sorbic Acid and Potassium Sorbate on the External Secretion of the Pancreas (Subacute Experiment)

Key: as above

Figure 7 Effect of 0.5 and 1.5% Sorbic Acid on the External Secretion of the Pancreas (Chronic Experiment)

Key: as above.

In the subacute feeding experiment (Fig.5), under the effect of 0.25% sorbic acid or potassium sorbate the amount of pancreas liquid and the total protein content rose significantly, also the activity of all of the enzymes. with the exception of amylase. The feeding of 2% sorbic acid or potassium sorbate for 4 weeks (Fig.6) resulted in a decrease in the chimotrypsin and amylase activities, in addition, potassium sorbate lead to a decline in the pancreas liquid and the lipase activity.

Figure 8 Effect of 0.5 and 1.5% Sorbic Acid of the Serum Enzyme Activity (Chronic Experiment)

The absorption of 0.5% sorbic acid (Fig. 7), given chronically, caused a slight increase in all cases, except chimotrypsin and lipase, while under the effect of 1.5% sorbic acid all enzyme activities rose, however, the deviations are not significant. It is seen in fig. 8 that the activity of serum chimotrypsin and lipase is practically unchanged. No change was found in the body and organ weights and the liver function test and histology did not show pathological changes.

Discussion

The experiments showed that certain preservatives caused deviations in the external secretion of the pancreas. In the case of the single dose (acute experiment), the changes observed demonstrate whether the fact whether the substance under investigation effected the external secretion of the pancreas. Specifically, this is true in the present experiments with respect to the deviation found in the external pancreas secretion under the effect of fresh

and decomposed pyrocarbonic acid diethylester. This lustifies the conclusion that -- due to its pancreas function reducing effect -- the undissociated pyrocarbonic diethylester must be considered harmful to health. After adequate storage, however, the substance in its dissociated state, enhances secretion and this supersecretion in itself (in the absence of other pathological findings) is harmless.

The acute effect of sodium benzoate, sodium salicylate and sorbic acid or potassium sorbate resulted only is slight decreasing trends in certain parameters (pancreas liquid, trypsin and chimotrypsin activites), in addition, under the effect of sorbic acid the activity of certain enzymes increased. These deviations emphasize the need for further studies.

The magnitude and direction of the variation in the pancreas function can be estimated from the results of the experiments (subacute) with administrations for 4 weeks, Under the effect of sodium benzoate, in agreement with the acute experiment, only in the activity of chimotrypsin was found a decrease. T he feeding of sodium salicylate, on the other hand, lead to decreases in all parameters, except the lipase activity; this indicates the almost complete inhibition of the external function of the pancreas. In connection with in vitro enzyme inhibition experiments, Bartlet /23/ found that benzoic acid in a concentration of 10^{-2} mole did not cause inhibition, among others, in pepsin activity. Land /9/ mentions that benzoic acid does not specifically inhibit chimotrypsin, but that under the effect of molar concentrations higher than 0.1, a nonspecific "salt" effect occurs, In the in vivo experiment of the authors, the administration of 2% sodium benzoate reduced the activity of chimotrypsin, however, the mechanism of the reduction was not investigated. Among the pharmacological effects of salicylates, as mentioned above, the irritation of the mucous membranes of the stomach and the inhibition of pepsin, are well known. The resulting digestive complaints may be explained by the reduction in the external secretion function of the pancreas. It is also known that both benzoates and salicylates in vitro, have a competitive

inhibiting effect on 1-amino acid oxidase. These data, and the results of the authors concerning the reduction of the activity of protein decomposing enzymes, make it liketo in that the intermediate metabolism of the proteins the salicylates, above a certain concentration the benzoates also, exercise an interfering activity.

In the subacute experiment, the effect of the consumption of 0.25% sorbic acid or potassium sorbate increased the external secretion of the pancreas; the cause of this probably is the small excess in the supply of fatty acids, to which the pancreas adapted itself. The consumption of the preservative for the same period of time but in an amount ten times as great resulted in a different reaction. It caused a reduction in certain parameters (chimotrypsin, amylase activity). Under the effect of potassium sorbate the amount of liquid secreted by the pancreas and the lipase activity also decreased slightly. These results indicate that the 2% preservative -- considered harmless in the past -- already to a certain extent affects the external secreation of the pancreas and cautions that constant consumption may lead to other damage (digestive, absorption disturbances);this, however, has not been investigated at the 2% level. It should be mentioned that the administration of fatty acid in a 2% concentration reduced the amylase activity. Several publications report the occurrence of enzyme responses independently of the consumption of certain nutritive components /25, 26/. In the present experiments this may be the case, or else, it may be a manifestation of the enzyme inhibiting effect of the fatty acid administered in large amounts (2%); according to these experiments, this effect appears only above a certain level.

In the subacute experiments, the amounts, which are 15-20 times the maximum concentrations permitted in products intended for human consumption, represent a necessary safety limit. The results support the prohibition of salicylic acid for use in the food industry, and reinforce the reservations which accompany the widespread use benzoic acid and its salts /27/.

Sorbic acid (chronic experiment) fed for 22 weeks in concentrations of

0.5 and 1.5% of the feed, increased the external secretion of the pancreas. This result must again be interpreted as an adaptation to sorbic acid consumption, because in the chronic experiment, no damage was observed as indicated by the body and organ weights, the liver function test and by histology. Serum enzyme activities did not increase either. It follows from the results that the safety limit of sorbic acid with respect to the external secretion function of the pancreas should be set at 1.5%, which is 15 times the maximum level permitted for human consumption. The experiments indicate that in the case of the chronic and subacute administration of preservatives the pancreas reacts sensitively to deviations in the amount of the various substances and that if a certain limit is exceeded, it signals -- prior to the appearance of other symptoms -- disturbances in the external secretion function of the pancreas and potential other consequences.

Summary:

Based on experiments with rats, the authors found that

1. the investigation of the external secretion function of the pancreas is capable of detecting early harmful effects of preservatives found "harmless" by the different methods found in the literature;

2. while in the case of the administration of a single dose (acute experiments) demonstrates the potential pancreas effect of the preservative under investigation, subacute and chronic experiments reveal the direction and magnitude of changes caused by the substance;

3. among the preservatives in the acute experiments, pyrocarbonic acid diethylester (0.32 g/kg) proved to be harmful to health, in the fresh undissociates state, due to the significant reduction of the external secretion of the pancreas; following storage, however, in the decomposed condition, it did not cause such a reduction, Under the acute (0.6 g/kg) and subacute (2%, 4 weeks) effect of sodium benzoate only the activity of chimotrypsin declined, while sodium salicylate (acute experiment: 0.32 g/kg) particularly in the

subacute experiment (2%, 4 weeks) completely inhibited the external secretion of the pancreas. Under the effect of sorbic acid and potassium sorbate (acute: 2 g/kg) the total protein content and chimotrypsin activity of the pancreas liquid declined slightly, while the lipase and amylase activities increased, in the subacute experiment (0.25%, 4 weeks), and the chronic experiment (0.5% and 1.5%, 22 weeks) the preservative increased the external secretion of the pancrease; subacute administration of 2% partially reduced the function.

References: Copy

(Translated by Carl Demrick Associates,Inc/LR/t)

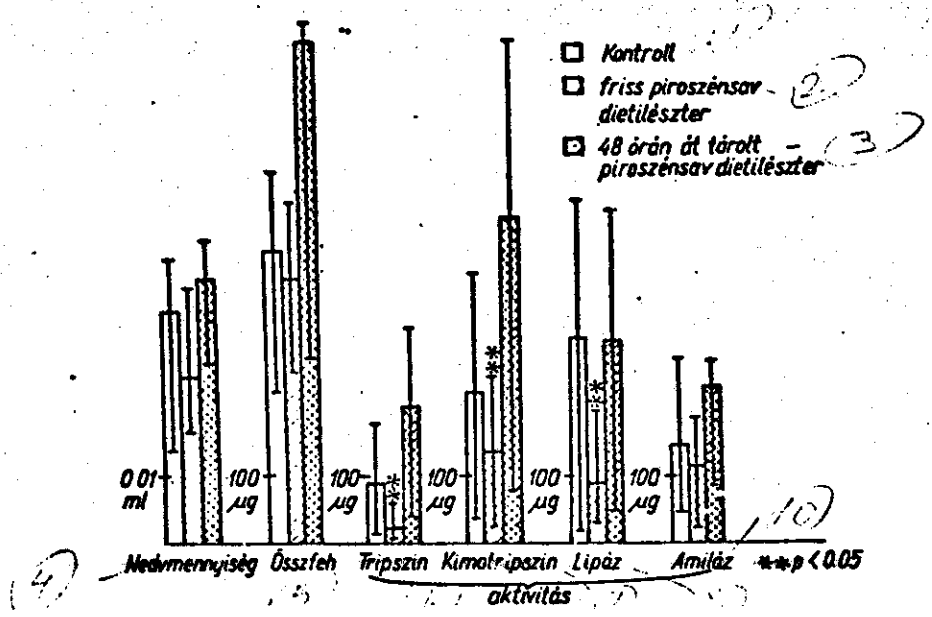

Figure 1

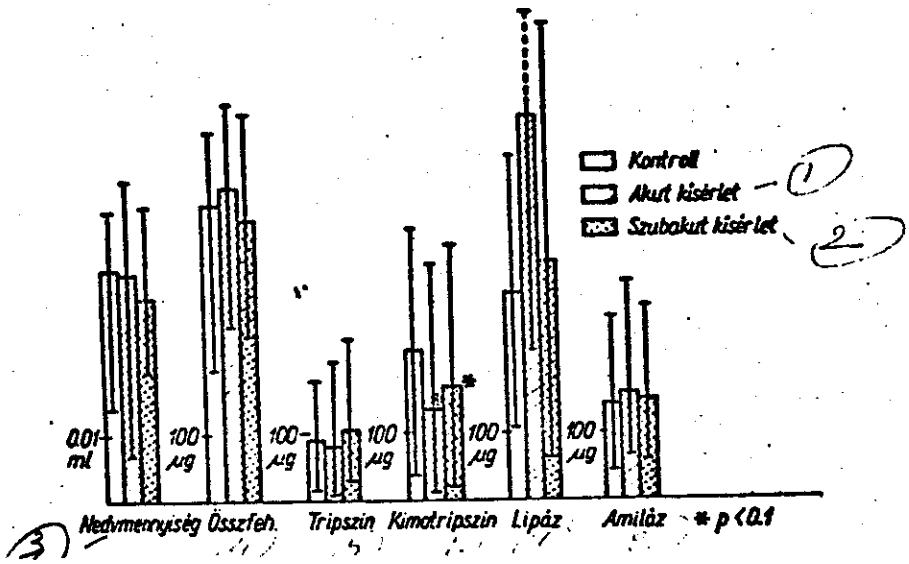

Figure 2

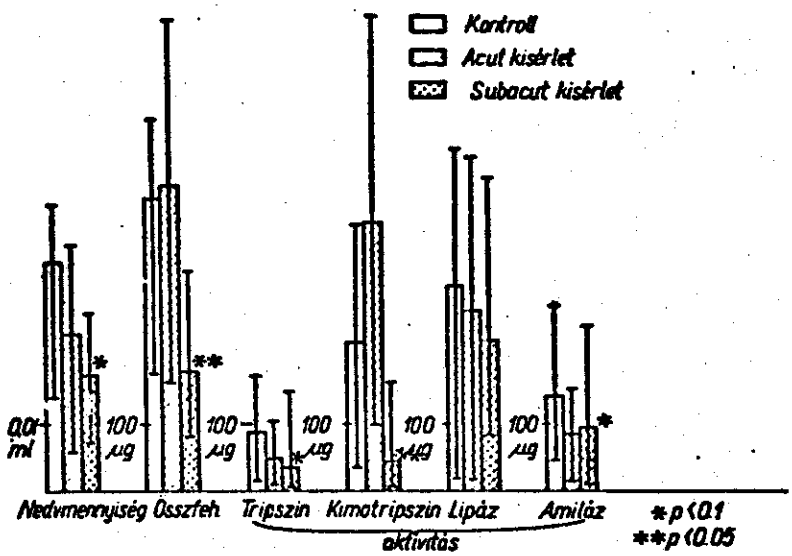

Figure 3

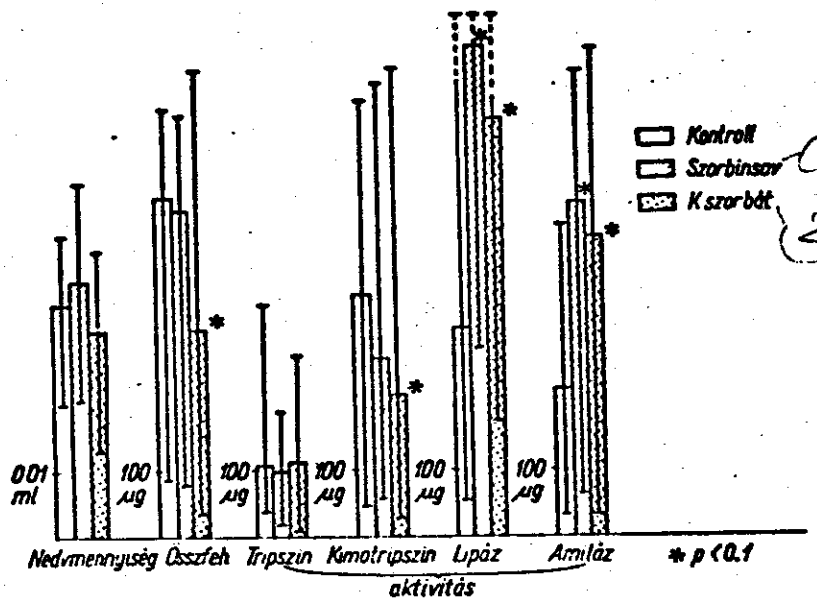

Figure 4

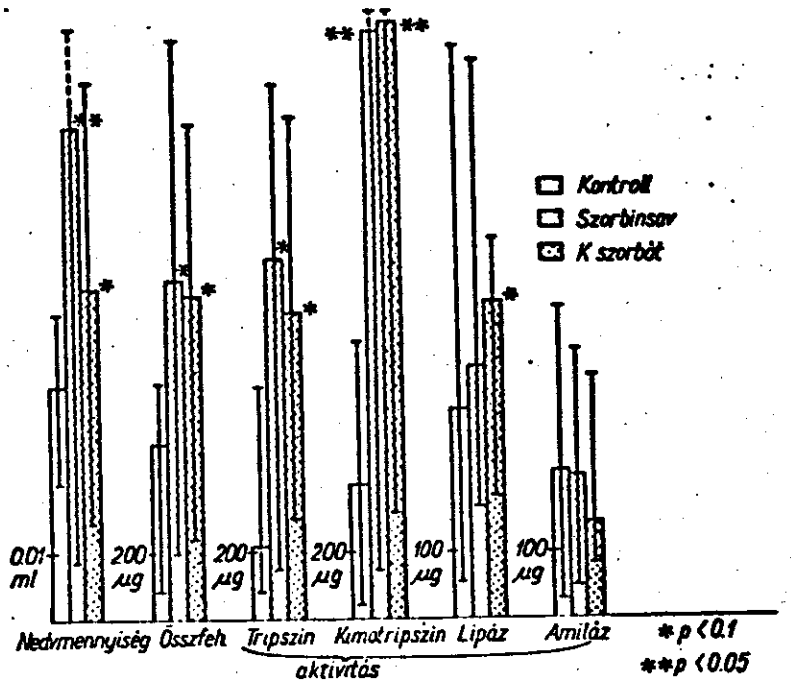

Figure 5

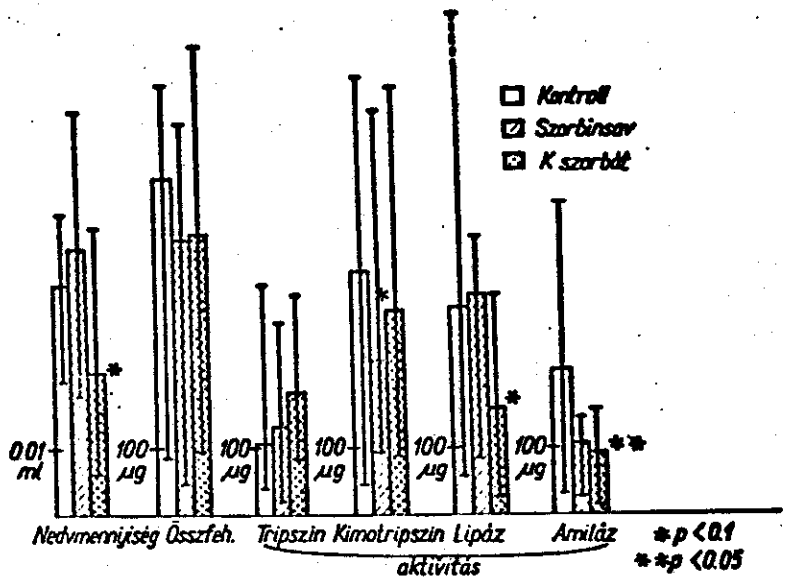

Figure 6

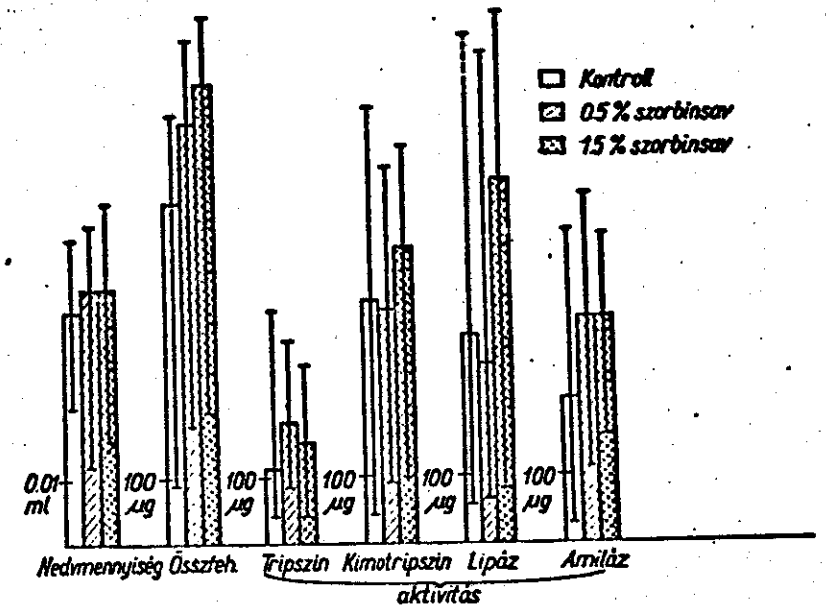

Figure 7

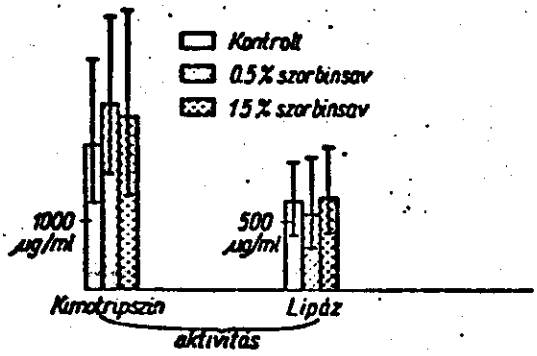
Kontroll
0.5% szorbinsav
1.5% szorbinsav
1000 µg/ml
500 µg/ml
Kimotripszin
Lipáz
aktivitás

Figure 8

Kísérletes Orvostudomány 20, 624—632. 1968.

Országos Élelmezés- és Táplálkozástudományi Intézet

Konzerválószerek hatása a pancreas külső secretiós működésére

J. ZSINKA ÁGNES és KEMÉNY TIBOR

Kézirat érkezett: 1968. január 4-én

Az élelmiszerekkel a szervezetbe jutó idegen anyagok között jelentős szerepet játszanak a konzerválószerek, amelyeket mind az élelmiszeriparban, mind a háztartásokban széles körben és rendszeresen alkalmaznak. Kiterjedt használatuk különösképpen jelentőssé válik dietetikai vonatkozásban, a betegélelmezésben, illetve egyes diétás készítményekben való alkalmazhatóságuk megítélésének körülményessége miatt. Élelmiszeripari felhasználásukat élelmezésegészségügyi elbírálásuk alapján szabályozzák; ez a szabályozás azonban országonként különböző. Az eltérő vélemények kialakulásának fő oka az, hogy az egyes konzerválószerek megítéléséhez rendelkezésre álló adatok különböző eredetűek: kis részük embereken végzett megfigyelésekből, túlnyomó részük viszont állatkísérletekből származik. Az állatkísérletekben alkalmazott módszerek pedig általában különbözők és így gyakran az eredmények sem egybehangzóak. Emiatt olyan általánosan használható állatkísérletes módszer alkalmazására lenne szükség, amellyel a különféle konzerváló anyagok ártalmatlan, vagy káros hatásáról és e hatások irányáról aránylag rövid idő alatt felvilágosítást szerezhetünk.

A fentiek alapján a különböző physiologiai hatásokra érzékenyen reagáló pancreas külső secretiós működését, illetőleg annak változását vizsgáltuk meg különféle konzerválószerek vonatkozásában. Kísérleteink célja tehát kettős volt: egyrészt a pancreas-testnek a vázolt célra való alkalmasságát, másrészt egyes konzerválószerek pancreas-functiót befolyásoló hatását kívántuk megállapítani.

A következő konzerválószerekkel végeztünk kísérleteket:

1. Az élelmiszeripar által bevezetésre javasolt *piroszénsavdietilészter* (Baycovin) főként gyümölcslevek és borok tartósítására alkalmas. Szúrós szagú, a nyálkahártyát izgató hatású anyag; állás, tárolás közben etanollá és széndioxiddá bomlik el. A bomlás sebessége az etanol és széndioxid koncentrációjának növekedésével csökken, s függ a hőmérséklettől és a pH-tól is. Ezért nagy körültekintést igényel a piroszénsavdietilészter adagolása és felhasználása között eltelt idő megállapítása.

A piroszénsavdietilészter orális LD_{50}-re patkányra vonatkozólag 1,57—1,39 g/testsúly kg (1), HECHT (2) szerint 1,1 ml. Ez utóbbi szerző kísérletében 1,5—2,5 ml/testsúly kg mennyiségek 12 órán belül mozgásgátlás és légszomj fellépése közben elhullásokat okoztak. Subacut kísérletében az LD_{50} 1/4 részének, azaz testsúly kg-ként 0,25 ml-nek, 4 hónapos, hetenként egyszeri adagolására a patkányok táplálékfelvétele és testsúlya csökkent. *Bornmann* (1) közölte, hogy 30 napig adagolt 2%-os friss piroszénsavdietilészter hasonló tüneteket okozott; 0,5%-ban való fogyasztása azonban nem idézett elő károsodást.

2. A *benzoesavat*, főként pedig *nátriumsóját* az élelmiszeriparban kiterjedten használják gyümölcs, főzelék és egyes édesipari készítmények konzerválására. Benzoesav (ortooxi-szalicilsav) nemcsak kívülről juthat a szervezetbe, hanem physiologiásan is képződik az intermediaer anyagcsere során, ahol glikokollhoz és glukuronsavhoz kapcsolódik, s hippursav formájában a vizelet útján választódik ki.

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Toxicitásának vizsgálata során megállapították, hogy a benzoesav orális LD_{50}-e patkányon 2—3 g/testsúly kg körül van (3). Chronikus fogyasztása embereken 0,5 és 1 g/nap dosisban semmiféle tünetet nem idézett elő (4), sőt a 12 g/nap dosisnak sem volt káros hatása (5). Az állatkísérletek eredményei szerint 2% nátriumbenzoát 3 hónapig tartó etetés folyamán, 35%-os kazein ellátás esetén nem okozott kóros elváltozásokat (6), 15% kazeint tartalmazó takarmányfogyasztás esetében azonban a szaporodást erősen zavarta és elhullásokat idézett elő (7). 20% kazein-tartalmú étrend 90 napos fogyasztásakor 1—4% nátriumbenzoát adagolás elhullást nem okozott (8). *Lang* (9) a kritikus határt 1—2% között állapította meg. A nátriumbenzoát konzerválószerként való felhasználása hazánkban készítményenként legfeljebb 0,15%-os mennyiségben engedélyezett.

3. A *szalicilsav*, illetve *nátriumsójának* használatát az élelmiszeriparban számos országban, köztük hazánkban is betiltották, de a háztartások zöme még ma is használja konzerválószerként. Ismert farmakologiai hatásain kívül a gyomornyálkahártyára gyakorolt izgató hatását, valamint pepszin aktivitásgátló hatását is leírták. Ld_{50}-e patkányon, per os 1,6 g/testsúly kg (10).

4. A *szorbinsavat* és *sóit* előszeretettel használják konzerválószerként az *élelmiszeriparban, terméken 0,1%-os maximális koncentrációban. A szorbinsavnak kiváló konzerváló hatása mellett előnyös tulajdonsága, hogy nem rontja a kész termék ízét*, mert mint ötszénatomos, telítetlen zsírsav, a szervezetben a táplálék zsírsavaihoz hasonlóan metabolizálódik, s végül széndioxiddá és vízzé bomlik el (11). Toxicitásával — többek között — *Deuel* (12), *Ignatyev* (13), *Cremer* (14), *Lang* (15) és *Smith* (16) foglalkoztak. Megállapították, hogy a patkányok oralis LD_{50}-e 5—10 g/testsúly kg között van. Éhezés esetén az LD_{50} értéke csökken. A subacut és chronikus toxicitásra vonatkozó állatkísérletekben a takarmány szárazanyagának 5—8%-ánál kisebb mennyiségben való etetése az eddigiekben alkalmazott különféle módszerek eredményei szerint nem idézett elő kóros elváltozásokat. A szorbinsav 0,1, 0,5 és 5%-ban, 100 napos etetés során nem okozott elváltozásokat sem a szaporodásban, sem az elhullásban (17). A takarmány szárazanyagának 8%-ában etetett szorbinsav hatására a máj súlyának kismértékű növekedését tapasztalták (12).

Módszerek

Kísérleteinket összesen 272 db (kontroll: 60-, piroszénsavdietilészter: 32-, Na benzoát: 38-, Na szalicilát: 34-, szorbinsav és káliumszorbát: 108 db) saját tenyésztésű patkányon végeztük el. Az acut hatás megállapítására vonatkozó kísérletekben az irodalmi LD_{50}-nek kb. 1/5 részét, tehát piroszénsavdietilészterből 0,32 g/testsúly kg-nyi frissen feloldott, vagyis bomlatlan; valamint 48 órán át szobahőn tárolt ugyanilyen mennyiséget, nátriumbenzoátból 0,6 g/testsúly kg-ot, nátriumszalicilátból 0,32 g/testsúly kg-ot, és szorbinsavból, valamint szorbinsavra számított káliumszorbátból 2 g/testsúly kg-ot adtunk be a patkányoknak gyomor-sondán át, egy alkalommal. A kontrollok azonos mennyiségű oldószert, vagyis desztillált vizet kaptak ugyancsak gyomor-sondán át. A nátriumbenzoát, nátriumszalicilát és szorbinsav, valamint káliumszorbát hosszabb ideig tartó (subacut) adagolására bekövetkező változásokat is megvizsgáltuk. A normál takarmányhoz (18% fehérjetartalom) 2%-ban kevertük a nátriumbenzoátot és nátriumszalicilátot; szorbinsavból és káliumszorbátból pedig 0,25 és 2%-ot tartalmazó takarmányt készítettünk, melyeket a patkányok 4 hétig fogyasztottak. A szorbinsav hatását chronikus kísérletben, 0,5 és 1,5%-os mennyiségek 22 hétig tartó adagolása esetén is megvizsgáltuk.

Mind az acut, mind a subacut és chronikus adagolás befejezésekor acut pancreas-fistulás műtét útján (18) két és fél órán keresztül (sekretin és karba-

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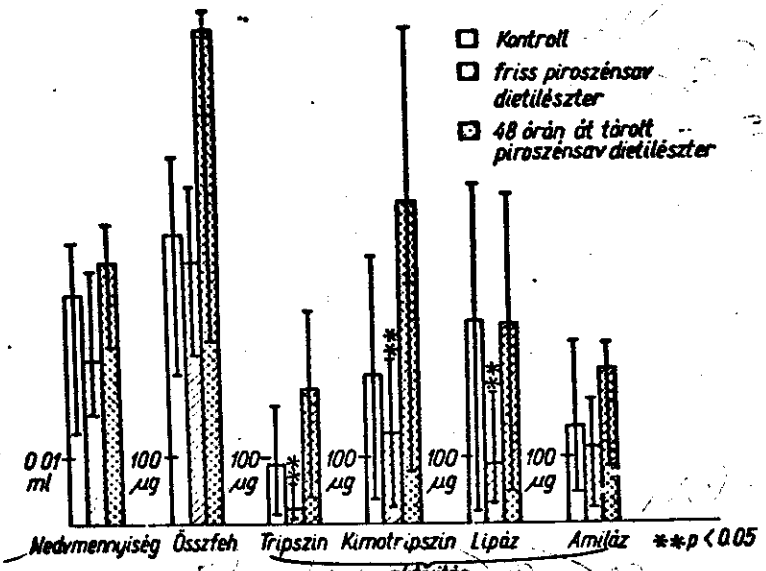
mylcholin adása nélkül) gyűjtöttük jeges hűtés mellett a pancreas nedvet. Mennyiségének lemérése után meghatároztuk a pancreas-nedv összfehérje-tartalmát (19), valamint tripszin-, kimotripszin-, lipáz- (20) és amiláz- (21) aktivitását. A tripszin aktivitását a substratból lehasított béta-naftilamin, a kimotripszin és lipáz aktivitását béta-naftol, az amiláz aktivitását keményítő μg-okban fejeztük ki, és a 60 perc alatt kiválasztott pancreas-nedv mennyiségekre számítottuk át. A kontroll-csoportokhoz viszonyított eltéréseket a Student-féle T-teszttel, ill. ahol arra lehetőség volt, a DD próbával értékeltük ki. A chronikus kísérlet tartama alatt ellenőriztük az állatok súlyát és a patkányok egy részénél máj-functiós (22) próbát hajtottunk végre, valamint meghatároztuk a serum lipáz és kimotripszin aktivitását is. Végül a patkányok elvéreztetése után szerveik súlyát lemértük és azokat szövettanilag feldolgoztuk.

Eredmények

Az 1. ábrán látható, hogy a piroszénsavdietilészter bomlatlan állapotban minden általunk vizsgált mutatóban csökkenést okozott, ez legjelentősebb volt a tripszin, kimotripszin és a lipáz aktivitásában. A 48 órán át tárolt piroszénsavdietilészter a pancreas külső secretiós működését nem csökkentette, inkább fokozta azt. A 2. ábrán a nátriumbenzoát hatására kialakuló helyzetet ábrázoltuk. Mint látható, egyedül a kimotripszin aktivitásában mutatkozott csökkenés, emelett az akut kísérletben emelkedett a lipáz aktivitása, ez az emelkedés azonban (a nagy szórás miatt) nem szignifikáns. A 3. ábrán látható, hogy nátriumszalicilát acut hatására a pancreas-nedv mennyisége, valamint a tripszin és amiláz aktivitása csökkenő tendenciát mutatott. A subacut adagolás hatására a lipáz aktivitás kivételével minden esetben csökkenést találtunk. Kifejezett csökkenés volt észlelhető az összfehérje-tartalomban és a kimotripszin aktivitásban. A 4. ábrán tüntettük fel az egyetlen adag (akut kísérlet) szorbinsav és



1. ábra. Piroszénsavdietilészter hatása a pancreas külső secretiós működésére (acut kísérlet)

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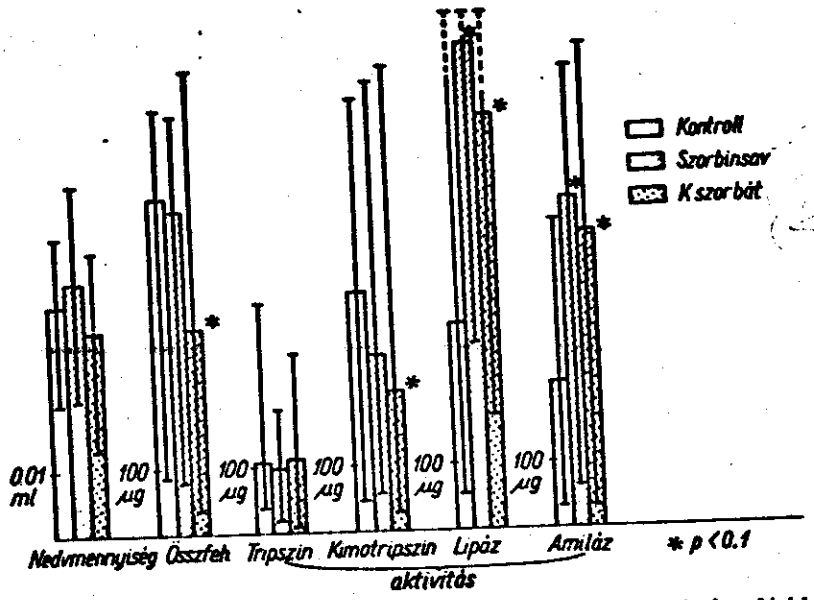
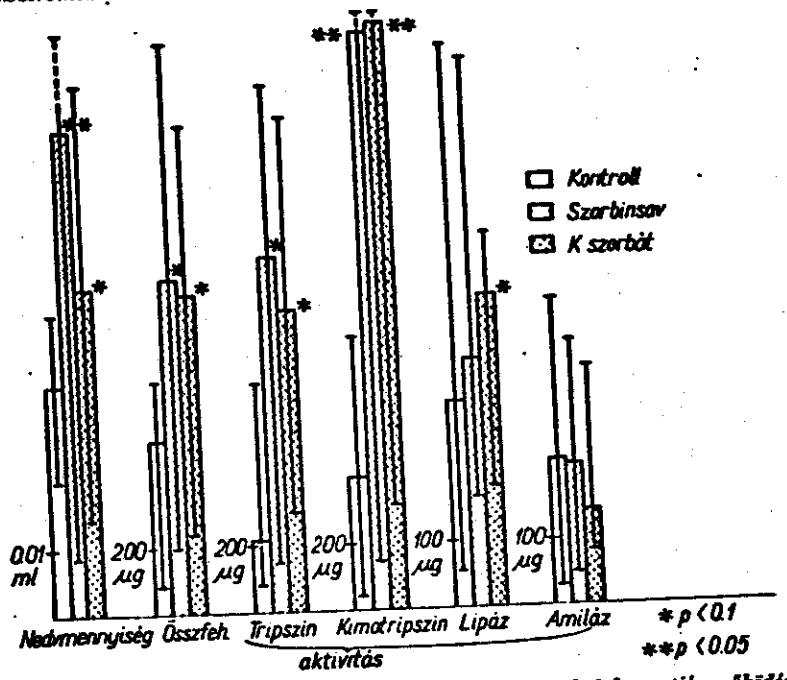


4ábra. Szorbinsav és káliumszorbát hatása a pancreas külső secretiós működésére (acutkisérlet)



5. ábra. 0,25% szorbinsav és káliumszorbát hatása a pancreas külső secretiós működésére (subacut kisérlet)

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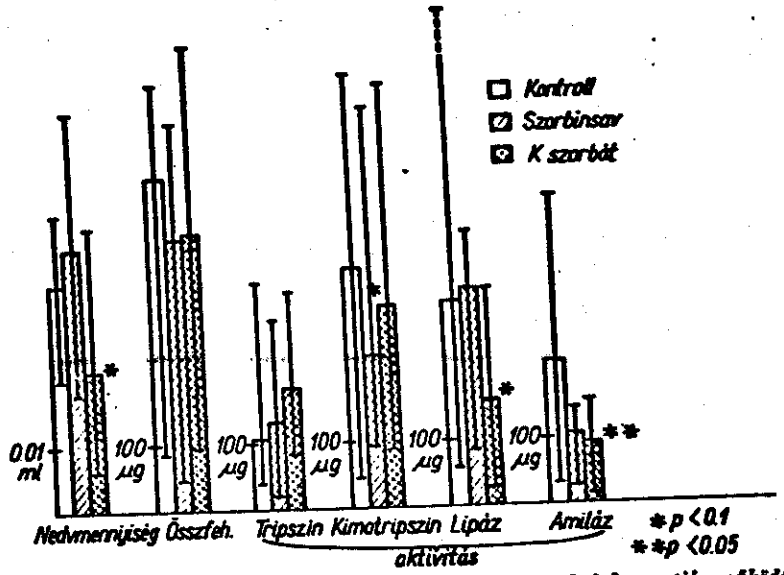
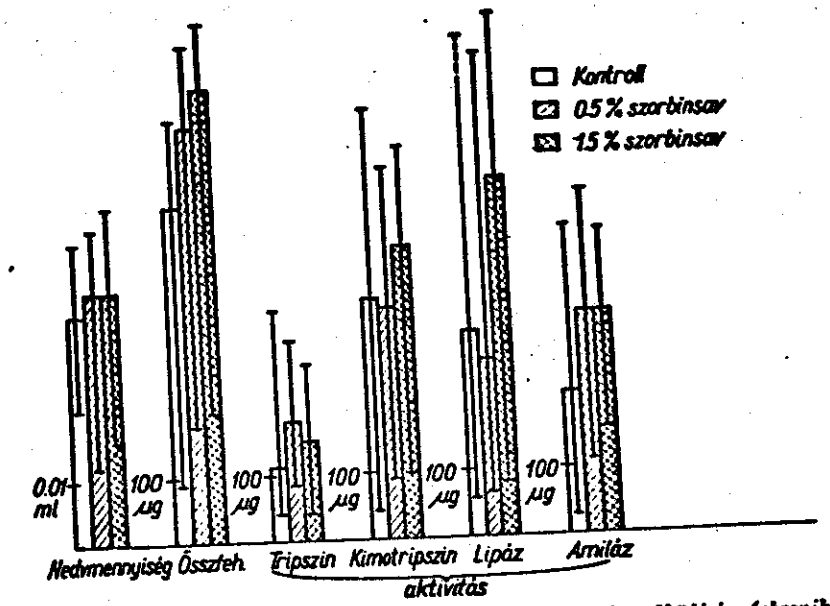


6. ábra. 2% szorbinsav és káliumszorbát hatása a pancreas külső secretiós működésére (subacut kísérlet)



7. ábra. 0,5 és 1,5% szorbinsav hatása a pancreas külső secretiós működésére (chronikus kísérlet)

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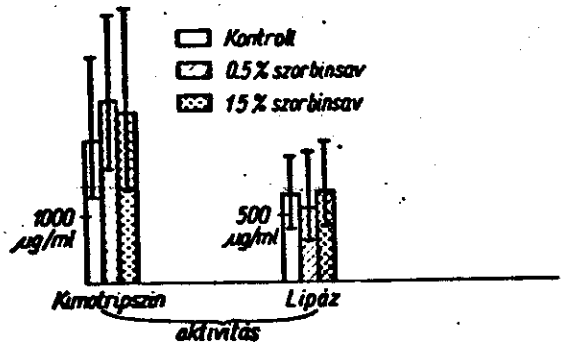
A subacut etetési kísérletben (lásd 5. ábra) 0,25% szorbinsav, illetve káliumszorbát adagolás hatására szignifikánsan emelkedett a pancreas-nedv mennyisége és összfehérjetartalma, valamint az amiláz kivételével minden enzym aktivitása. 2% szorbinsav, illetve káliumszorbát 4 hétig tartó etetése (lásd 6. ábra) esetén csökkent a kimotripszin és amiláz aktivitása, továbbá káliumszorbát hatására csökkent még a pancreas-nedv mennyisége és annak lipáz aktivitása is.



8. ábra. 0,5 és 1,5% szorbinsav hatása a sérum enzym-aktivitására (chronikus kísérlet)

A chronikusan etetett (lásd 7. ábra) 0,5% szorbinsav fogyasztása a kimotripszin és lipáz aktivitás kivételével minden esetben kismértékű növekedést idézett elő. 1,5% szorbinsav hatására pedig minden enzym aktivitása emelkedett, matematikailag azonban az eltérések nem szignifikánsak. A 8. ábrán látható, hogy a serum kimotripszin és lipáz aktivitása gyakorlatilag változatlan volt. Nem találtunk eltérést a test- és szervsúlyokban sem; s a máj-functiós próba, valamint a szövettani leletek eredményei sem mutattak kóros elváltozást egyik csoportban sem.

Megbeszélés

Kísérleteink szerint a pancreas külső secretiós működésében egyes konzerválószerek hatására eltérés mutatkozott. Az egyetlen dosis esetén (acut kísérlet) létrejött változások alapján megállapíthattuk, hogy a vizsgált anyag hatásos, vagy hatástalan volt-e a pancreas külső secretiós működésére. Kísérletünkben erre utalt különösképpen a bomlatlan és az elbomlott piroszénsavdietilészter alkalmazásakor kialakuló eltérés a pancreas-functióban. Ez esetben azt a következtetést vonhattuk le, hogy a bomlatlan piroszénsavdietilészter — pancreas-functiót csökkentő hatása miatt — egészségkárosítónak tekinthető. Megfelelő tárolás után, elbomlott állapotban azonban a pancreas külső secretiós működését inkább fokozta s ez a supersecretio önmagában (egyéb kóros leletek nélkül), ártalmatlannak minősül.

A nátriumbenzoát, nátriumszalicilát és szorbinsav, illetve káliumszorbát akut hatására csak kismértékű csökkenő tendenciát találtunk egyes mutatókban (pancreas-nedv mennyisége, tripszin, illetve kimotripszin aktivitása) emellett szorbinsav hatására egyes enzymek aktivitása emelkedett. Ezek az eltérések a további vizsgálatok szükségességére hívták fel a figyelmet.

A pancreas-functio változásának mértékére és irányára a konzerválószerek 4 hétig tartó adagolása esetén végzett (subacut) kísérletek eredményeiből következtethettünk. Nátriumbenzoát hatására, az akut kísérlettel megegyezően,

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csak a kimotripszin aktivitásában tapasztaltunk csökkenést. Nátriumszalicilát etetése azonban a lipáz aktivitás kivételével minden mutatóban csökkenést idézett elő, amely a pancreas külső secretiós működésének csaknem teljes akadályoztatását jelezte. A benzoesavval, illetőleg benzoáttal kapcsolatos in vitro, enzym-gátlásra irányuló kísérletek során *Bartlett* (23) azt tapasztalta, hogy a benzoesav 10^{-2} mol. koncentrációban nem okozott gátlást — többek között a pepszin aktivitásában. *Lang* (9) megemlíti, hogy a benzoesav a kimotripszint nem specifikusan gátolja, hanem a 0,1 vagy ennél magasabb mol. koncentráció hatására egy nem specifikus „sóhatás" érvényesül. In vivo kísérletünkben 2% nátriumbenzoát adagolása a kimotripszin aktivitását csökkentette, a csökkenés mechanizmusát azonban nem vizsgáltuk. A szalicilátok farmakologiai hatásai között, mint említettük, azok gyomornyálkahártyát izgató, valamint pepszinaktivitást gátló tulajdonságai jól ismertek. Az emésztési panaszok fellépését eredményeink alapján a pancreas külső secretiós működésének csökkenése is magyarázza. Ismeretes az is, hogy mind a benzoátok, mind a szalicilátok in vitro, competitiv gátló hatást fejtenek ki az l-aminosavoxidázokra (24), a benzoesav pedig emelett a d-amonosavoxidáz aktivitását is gátolja. Ezek az adatok, valamint saját, in vivo kísérleteinkben észlelt, a fehérjebontó enzymek aktivitásának csökkenésére utaló adataink valószínűsítik, hogy a fehérjék intermediaer anyagcseréjében a szalicilátok, sőt bizonyos koncentráción felüli mennyiségben a benzoátok is zavart okoznak.

A subacut kísérletben 0,25% szorbinsav, illetőleg káliumszorbát fogyasztásának hatására a pancreas külső secretiója egészében fokozódott, amelynek oka valószínűleg a normál ellátáson felüli, kis mennyiségű zsírsav-többletbevitel volt, melyhez a pancreas adaptálódott. A hasonló ideig tartó, de kb. tízszeres mennyiségű konzerválószer fogyasztása azonban már más reactiót váltott ki. Egyes mutatókban ugyanis (kimotripszin, amiláz aktivitás) csökkenést idézett elő. Kismértékben csökkent továbbá káliumszorbát hatására a pancreas által kiválasztott nedv mennyisége és annak lipáz aktivitása is. Ezek az eredmények azt jelzik, hogy — az eddigiekben hatástalannak tartott — 2%-os konzerválószer-szint már bizonyos mértékig károsan befolyásolta a pancreas külső secretióját s arra figyelmeztet, hogy tartós fogyasztása esetleg egyéb károsodásokat (emésztési, felszívódási zavarokat) is okozhat, erre vonatkozólag azonban — 2%-os szinten — nem végeztünk vizsgálatokat. Említésre tarthat számot az az eredmény, hogy a 2%-os mennyiségben adagolt zsírsav bevitel hatására az amiláz aktivitása csökkent. Több közlemény említi az egyes tápanyag alkotórészek fogyasztásától független enzym-válaszok felléptét (25, 26). Kísérletünkben vagy ezzel a hatással kell számolnunk, vagy pedig a nagyobb mennyiségben (2%-ban) etetett zsírsav enzym-aktivitásgátló hatásának megjelenésével, amely eredményeink szerint csak bizonyos szinten felül érvényesül.

Az általunk végzett subacut kísérletekben az emberi fogyasztásra élelmiszeripari termékekként maximálisan engedélyezett mennyiségek 15—20-szorosa a szükséges biztonsági határt jelenti. Az eredmények alátámasztják a szalicilsav élelmiszeripari célokra való felhasználásának eltiltását, valamint megerősítik a benzoesav, illetve sóinak kiterjedt használatával kapcsolatos aggályokat (27).

A takarmány 0,5 és 1,5%-ában 22 hétig etetett szorbinsav (chronikus kísérlet) fokozta a pancreas külső secretiós működését. Ezt az eredményt is a szorbinsavfogyasztáshoz való adaptatióként kell értékelnünk, minthogy a chronikus kísérletben sem a test- és szervsúlyok, sem a máj-functio és a szövettani leletek nem jeleztek károsodást, valamint a serumban található enzym-aktivitasok sem növekedtek. Az eredményekből következik, hogy a szorbinsavnak a pancreas külső secretiós működésére vonatkoztatott biztonsági szintjét 1,5%-ban szab-

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hatjuk meg, ami az emberi fogyasztás maximális engedélyezett szintjének 15-szörös mennyiségét jelenti.

Kísérleteink arra utalnak, hogy a subacut és chronikus időtartamú konzerválószer adagolás során a pancreas-test érzékenyen reagál a vizsgált anyagok mennyiségeinek eltérésére a bizonyos határon felüli bevitel esetén — az egyéb tünetek megjelenése előtt — felhívja a figyelmet a pancreas külső secretiós működésének zavarára s így annak esetleges következményeire.

Összefoglalás

A szerzők patkánykísérleteik eredményei alapján megállapították, hogy

1. a pancreas külső secretiós működésének vizsgálatával az irodalomban található különböző módszerek szerint „hatástalan"-nak tartott konzerválószer mennyiségek korai károsító hatása kimutatható;

2. míg az egyetlen dosis (akut kísérletek) beadása esetén a vizsgált konzerválószer esetleges pancreas-functiót befolyásoló hatását lehet megállapítani, addig a subacut és chronikus időtartamú kísérletek eredményei az illető anyag hatására létrejövő elváltozások irányát és mértékét jelzik;

3. a konzerválószerek közül acut kísérletben a piroszénsavdietilészter (0,32 g/testsúly kg) friss, vagyis bomlatlan állapotban egészségkárosító hatásúnak bizonyult a pancreas külső secretiójának szignifikáns csökkentése miatt; tárolás után, elbomlott állapotban azonban a pancreas külső secretiós működésében csökkenést nem okozott. A nátriumbenzoát akut (0,6 g/testsúly kg) és subacut (2%, 4 hétig etetve) hatására csak a kimotripszin aktivitása csökkent, míg a nátriumszalicilát (akut kísérlet: 0,32 g/testsúly kg) — főként a subacut kísérletben (2%, 4 hétig etetve) a pancreas külső secretiós működését egészében akadályozta. A szorbinsav és káliumszorbát akut (2 g/testsúly kg) hatására enyhén csökkent a pancreas-nedv összfehérje-tartalma és kimotripszin aktivitása, egyidejűleg emelkedett a lipáz és amiláz aktivitása. A subacut kísérletben 0,25%-ban (4 hétig etetve), és a chronikus kísérletben 0,5-, valamint 1,5%-ban (22 héten át) adagolva, fokozta a pancreas külső secretióját; 2%-os szinten való subacut időtartamú adagolása azonban a pancreas külső secretiós működését részben csökkentette.

IRODALOM. 1. *Bornmann G. A.:* Archiv f. Tox., *19*, 69, 1961. — 2. *Hecht K.:* Ztschr. f. Lebensmitt. Ubtersuch. u. Forsch., *114*, 292, 1961. — 3. *Spector W. S.:* Handbook of Tox. Philadelphia, London, 1956. — 4. *Gerlach H.:* Phisiologische Wirkungen der Benzoesäure Wiesbaden 1909. — 5. *Waldo J. F.* et al.: Amer. J. Med. Sci., *217*, 563, 1949. — 6. *Griffith W. H.:* J. Biol. Chem., *82*, 415, 1929. — 7. *Meunier P.:* Bull. Soc. Chim. Biol. Paris, *31*, 1413, 1949. — 8. *Deuel H. J.* jr. et. al.: Food Res., *19*, 1, 1954. — 9. *Lang K.:* Ztschr. f. Lebensmittel Untersuch. u. Forsch., *112*, 394, 1960. — 10. *Hart I.:* Pharm. Exp. Ther., *38*, 9, 1930. — 11. *Deuel H. J.* et al.: Food Res., *19*, 13, 1954. — 12. *Deuel H. J., R. Alfin—Slater:* Food Res., *19*, 1, 1954. — 13. *Ignatyev A. D.:* Voproszü Pitanyija, *3*, 61, 1960. — 14. *Cremer H. D.:* Klin. Wschr., *37*, 304, 1959. — 15. *Lang K.:* Arzneimitt. Forsch., *10*, 997, 1960. — 16. *Smyth H. T., C. P. Carpenter:* J. Ind. Hyg. Toxikcol., *30*, 63, 1948. — 17. *Kieckebursch W., K. Lang:* Arzneimitt. Forsch., *10*, 1001, 1960. — 18. *J. Zsinka Á., Faur N., Kemény T., Véghelyi P.:* Kísérletes Orvostudomány, *16*, 475, 1964. — 19. *Lowry O. H.:* J. Biol. Chem., *193*, 265, 1951. — 20. *Schön H.*, et al.: Klin. Wschr., *39*, 217, 1961. — 21. *Schmith B. W., R. J. H. Roe:* J. Biol. Chem., *17*, 53, 1949. — 22. *Frommer J.:* Proc. Soc. Exp. Biol. Med., *86*, 567, 1954. — 23. *Bartlett A. R.:* J. Amer. Chem. Soc., *70*, 1010, 1948. — 24. *Kearney E. B.:* Arch. Biochem., *27*, 348, 1950. — 25. *Wang C. C., M. I. Grossmann:* Am. J. Phys., 164, 527, 1951. — 26. *Mages D. F., S. S. Hong:* Am. J. Phys., *197*, 27, 1959. — 27. *Ignatyev A. B.:* Voproszü Pitanyija, 23, 6, 1964. Voproszü Pitanyija, *3*, 61, 1960.

Й. Жинка Агнеш, Кемень Тибор: *Действие консервирующих средств на внешнюю секреторную деятельность пожелудочной железы.*

Авторы в экспериментах на крысах исследовали изменения внешней секреции под-

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желудочной железы под влиянием консервирующих средств и установили, что „панкреас-тест" является подходящим методом для определения раннего повреждающего эффекта различных консервирующих средств и для выявления степени и направления вредного действия.

В своих экспериментах авторы исследовали действие следующих веществ при остром, подостром и хроническом сроке их введения: диетилэстер пироугольной кислоты (Банковин), бензоат натрия, салицилат натрия, сорбиновая кислота и сорбат калия.

Á. J. Zsinka und T. Kemény: *Über die Wirkung von Konservierungsmitteln auf die Sekretionsfunktion des Pankreas.*

In Rattenversuchen wurde auf Grund der sich in der Sekretionsfunktion des Pankreas auf Wirkung von Konservierungsmitteln ausbildenden Veränderungen festgestellt, dass der Pankreastest für den Nachweis der frühen schädigenden Wirkung der verschiedenen Konservierungsmittel geeignet ist, weiterhin auch für die Bestimmung von Mass und Richtung dieser Wirkung. Im Laufe der Versuche wurden die auf Wirkung der akuten bzw. subakuten Verabreichung und der prolongierten Gabe von Baycovin, Natriumbenzoat, Natriumsalicylat, Sorbinsäure und Kaliumsorbat zustandekommenden Veränderungen untersucht.

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UDK (Universal Decimal Classification):

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Effect of Certain Food Preservaties on the Development of Malignant Processes in Mice

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The utilization of various chemical substances in the food industry has shown a pronounced growth. The possibility of the toxic and carcinogenic action of these additions to nutritional products, in the case of their prolonged entry into the human organism, has not been sufficiently studied.

Fruit, vegetables and berries are treated with sodium bisulfite for the purpose of safeguarding them from mold. Its permissible residual concentration is up to 300 milligrams to 1 kilogram. Benzoic acid is used, basically, for the treatment of the same products, and of the preserves prepared from them, with the purpose of preventing fermentation; its concentration does not exceed 0.2% of the product's weight. Both preservatives have already been utilized for close to half a century.

Until recently, sodium bisulfite and benzoic acid were considered to be relatively harmless, non-carcinogenic (Kiechebusch and Lang; Hosine; Lockett and Natoff). A number of papers have been published, however, that indicate a negative effect on the part of these substances on the human organism (Fitzhugh and coauthors; Morgan); in connection with this, in some countries (France), the application of benzoic acid has been forbidden, and the problem concerned with sulfurous compounds has been submitted for the consideration of the WHO (World Health Organization).

Sorbic acid and nisin represent new preservatives. Sorbic acid, which had been isolated for the first time from the rowan berry, has proved to be a very effective preservative. It represents a polyunsaturated fatty acid with 6 carbon atoms. According to the data in the literature (Lang), the addi-

tion of 5% sorbic acid to the diet of rats did not bring about any pathological changes, and the addition of 8% of the same substance (Deuel and coauthors) led to an increase in the weight of the liver. We have not disclosed any indications whatsoever in the literature pointing to the carcinogenic properties of sorbic acid.

The antibiotic nisin was isolated for the first time from lactic products. It represents a product of the vital activity of lactic streptococci, and has no medicinal significance, but it does reduce the resistivity of the spores of thermostable bacteria to heating; this makes it possible, on adding it to preserves, to reduce the level of sterilization and, at the same time, to increase the nutritive value of the preserves. There is an absence of data with regard to the carcinogenic capacity of nisin.

In studying the toxicity of benzoic acid in concentrations in which this substance is contained in the nutritive products of the human being, in mice there has been disclosed the lowest survival rate and the lowest gain in body weight, a reduction in the detoxication capacity of the liver, and the highest degree of expression of a negative effect on the organism of the animals during the action of cold and during the period of staying in a centrifuge (A.D. Ignat'ev).

The composite action of benzoic acid and sodium bisulfite has also brought in its wake a marked reduction in the survival rate of the mice, a diminution in the use of water and feed by them, a reduction in body weight and in endurance relative to physical burdening and hunger. There has also been noted a hypertrophy of some organs, particularly of the liver.

Sorbic acid proved to be non-toxic. Its introduction into the diet of the animals brought about higher indices than in the case of the control animals for the survival rate, increase in weight, utilization of feed, phagocytal activity, and so forth.

Nisin possessed the highest toxicity; it was manifested even in mixing it with sorbic acid. Along with this, there was also observed some antagonism on the part of the action of sorbic acid and of nisin. Basically, all the above-

mentioned indices came closer to the norm than in the case of the introduction of nisin alone, possibly, as a result of the favorable action of sorbic acid.

In all the experiments in which the mice received nisin alone, or nisin along with sorbic acid, there was disclosed a greater gain in their weight. In one series of experiments, adiposis was also established -- the body weight exceeded 40 grams in 44% of the experimental animals.

Cases of adiposis were likewise encountered among animals that had received sodium bisulfite jointly with benzoic acid; in such case, it was particularly pronounced in the case of the females (A. D. Ignat'ev).

In our investigations, use was made of 365 mice of box sexes. They were subdivided into 5 groups: four experimental and one control. All the animals received an identical diet, in the beginning, from natural products (according to the norms of the AMNSSSR (Academy of Medical Sciences of the U.S.S R.)), and after that, from briquetted feeds. The test animals had added to their feed the preservatives under study: 0.2% benzoic acid, 0.01% nisin, 0.2% sorbic acid or 0.4% sodium bisulfite (according to the weight of the diet). The doses of the preservatives corresponded to those that are introduced into the nutritive products of a human being. In special groups of mice we made a study of the composite action of a mixture of sodium bisulfite with benzoic acid and of a mixture of sorbic acid with nisin. The experimental animals, in the course of 3 months, received these preservatives with their food in an amount indicated above; following this, they and the control animals had transplanted in them, intraperitoneally, an ascitic Ehrlich cancer.

As is known, upon the administration, into the abdominal cavity of mice, of 50 - 80 million tumor cells, an ascitic Ehrlich cancer develops in 100% of the inoculated mice ((those having had the transplant)); the duration of their life, following the transplant, comprises 12 - 17 days. It has been established through our preliminaryobservations, that in the administration of a smaller number of tumor cells (1.2 - 1.25 millions cells), tumors develop in 70 - 100% of the mice; the duration of their life is somewhat longer (21 - 30 days).

For the purpose of establishing more clearly the effect of the indicated pre-

servatives on the development of an ascitic cancer, we made use of precisely such a method of inoculation (transplantation). Prior to the experiment, the mice were weighed and a measurement of their abdomen size was made. Subsequently, measurements were carried out every 4 days from the day of the transplant of the tumor and up to the death of the animals. The duration of observing them comprised 53 - 66 days; when this period of time had elapsed, all the animals that had survived were put to death and cut open. In all the mice (those that had perished and those that had been put to death), a measurement was made of the amount of asicitic liquid, and a note was made of the degree of its hemorrhaging tendency. The latter was designated in units in the following manner: + light-pink pigmentation -- 1 unit; red -- 2 units; and dark-red -- 3 units. The data thus obtained were treated statistically; the index of vitality (P) was determined by comparing the indices of the given group with the control group.

The data obtained are set forth in Tables 1 and 2.

As may be seen from Tables 1 and 2, tumors were more frequently transplanted in mice receiving sodium bisulfite and benzoic acid separately, and to a lesser degree where there was a joint administration of both substances. The shortest life span of mice following a transplantation of the tumor was found in mice that had received, separately, sodium bisulfite and benzoic acid; the volume of ascitic acid was the greatest in mice that had received sodium bisulfite.

Table 1

Results of the Transplant of an Ascitic Ehrlich Cancer in Mice That Had Received Preservatives

Preservative	Number of Mice	Duration of	Number of Tumors absolute	Number of Tumors %	P
Sodium Bisulfite	97	53	61	62.9±6.5	Illegible
Benzoic Acid	90	53	62	60.8±5.1	<0.001
Sodium bisulfite + benzoic acid	22	66	9	40.9±10.4	<0.01

Table 1 continued.

Sorbic acid	58	66	17	29.3±6.0
Sorbic Acid + nisin	49	66	15	30.6±6.1
Control	49	66	16	32.6±6.5

Table 2

Life Span of Mice with an Ascitic Ehrlich Cancer, the Mean Volume of Ascitic Liquid and the Degree of Its Hemorrhaging Tendency

Preservative	Beginning of Death in the	Mean Volume of the Ascitic Liquid (in milliliters)		Degree of (in units)
		M plus or	P	
Sodium Bisulfite	12	6.5±0.418	<0.002	2.7
Benzoic Acid	15	5.03±0.330	<0.001	2.5
Sodium bisulfite + benzoic acid	24	3.4±0.476	0.001	2.5
Sorbic Acid	21	5.06±0.626	>5	2.6
Sorbic Acid + nisin	21	4.09±0.446	>5	3.0
Control	24	5.03±0.86		2.5

((text continued)): The percent of tumors that had developed in mice having received sorbic acid, separately, or along with nisin, did not differ from the one that was observed in the control mice not having received preservatives, additionally. The life span of these mice, following transplantation, was negligibly shorter than that of the control ones; the volume of the ascitic liquid in mice that had received sorbic acid was the same as in the control ones, and in mice having received sorbic acid and nisin, negligibly less. The degree of hemorrhaging tendency on the part of the ascitic liquid, in arbitrary units, was identical in all thegroups.

Thus, the addition, to the diet, of sodium bisulfite and benzoic acid, was instrumental in producing a more intensive development of the ascitic Ehrlich cancer.

We also made a study of the effect of the body weight on the frequency of development of an ascitic Ehrlich cancer during the introduction of preservatives into the diet. As has been indicated above, upon introducing, into the diet of the experimental mice, sorbic acid and nisin,and in individual cases, sodium bisulfite, also, with benzoic acid, there was noted a tendency towards a marked increase in the body weight of the animals. In 40% of the mice, the body weight proved to be higher than 40 grams, and in individual cases, it attained 50 - 60 grams.

According to the date in the literature, in the event of mice adiposis, which was brought about by aurothioglucose, there is observed a more intensive development of malignant tumors. Thus, Wexler indicates that spontaneous tumors of the mammary gland developed earlier and more frequently in mice of the A line with an adiposis brought about by aurothioglucose.

The same thing is observed, likewise, in the relationship of the development of a spontaneous hepatoma in male mice of the C_3H line.

In our experiments, 90 mice received, over the course of 8 months, either a mixture of sorbic acid with nisin, or else sodium bisulfite with benzoic acid in doses indicated above. In view of the fact that adiposis was observed in varying percents of cases depending on the sex of the animal (more frequently in the cases of females), we included males and females in the experiment. Experiments were set up both for fattened mice as well as for mice having the customary weight. For this purpose, the mice were broken up into 9 groups, with 10 mice in each group (males and females of customary weight, males and females with an increased weight, and a control group).

The experiment had to furnish an answer to the question whether the introduction of a preservative, into the diet, is instrumental in a more intensive development of a malignant process, or whether, in fact, this effect represents a secondary one as the result of the adiposis brought about by the given preservative.

All the mice were administered, intraperitoneally, 1.2 - 1.25 million cells

of an ascitic Ehrlich cancer. We were not able to observe any differences in the development of a tumor in mice belonging to the different groups; nevertheless, the life span of the animals, and the volume of the ascitic liquid in them, were not identical. Thus, the life span of mice that had received a mixture of sodium bisulfite with benzoic acid was, on an average, somehwat shorter following the transplantation of a tumor (11 - 13 days), and the volume of the ascitic liquid -- somewhat greater (9 - 11 milliliters) in comparison with the remaining groups (in the control mice, it was, respectively, 15 days and 7 - 8 milliliters). The difference was not reliable statistically; however, the tendency that was manifested characterized a greater malignancy of an ascitic Ehrlich cancer in mice that had received sodium bisulfite along with benzoic acid. A similar effect, evidently, is not associated with the capacity of these preservatives to produce adiposis.

The beginning of the death of the mice, which characterized the intensity of development of the tumor, the volume of the ascitic liquid, and the degree of its hemorrhaging tendency are shown in Table 2.

Conclusions

1. In the transplantation of an ascitic Ehrlich cancer in mice that had received, with their feed, already previously utilized preservatives - sodium bisulfite and benzoic acid, the number of developed tumors was the least. The life span of these mice was the shortest, but the volume of the ascitic liquid was the greatest. This attests to the greater malignancy of the tumors that had developed in them, in comparison with the control mice.

2. The percent of developed tumors in mice that had received sorbic acid and nisin, and also, the degree of its malignancy, were the same as they were in the control mice.

Bibliography

1. Ignat'ev, A. D., Vopro. pit. (Problems in Nutrition), 1965, No. 3, p. 61.

2. Deuel, H. J., Alfin-Slater, R., Weie, C. S., et al., Food Res., 1954, v. 19, p. 1.

3. Fitzhugh, O. G., Knudsen, L. F., Nelson, A. A., J. Pharmacol. Exp. Ther., 1946, v. 86, p. 37.

4. Hosino, Cited by J. Hartwell, Survey of Compounds Which Have Been Tested for Carcinogenic Activity, Washington, 1951, p. 54.

5. Kieckebusch, W., Lang, K., Arzneimittel-Forsch, 1960, Bd 10, S. 1001.

6. Lang, K., Ibid., S. 997.

7. Lockett, M. F., Natoff, I. L., J. Pharmacol., 1960, v. 12, p. 488.

8. Morgan, A. F., Am. J. Publ. Hlth., 1935, v - 25, p. 328.

9. Waxter, S., Tabar, P., Melcher, L., Cancer Res., 1953, v. 13, p. 276.

10. Waxler, S., J. Nat. Cancer Inst., 1954, v. 14, p. 1253.

11. Idem., Stanford Med. Bull., 1957, v. 15, p. 26.

12. Idem., Am. J. Clin. Nutr., 1960, v. 8, p. 760.

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тик при термической обработке продуктов, содержащих его остат количества, безусловно, нуждаются в дополнительном изучении.

Решение этих вопросов должно идти с учетом возможного ра ного механизма связывания антибиотиков с белками тканей жи организма (в случае скармливания или введения антибиотика пар рально) и белками мяса, в котором уже произошли посмертные изм ния, если оно подвергается обработке растворами антибиотиков с це удлинения сроков его хранения.

ЛИТЕРАТУРА

Кивман Г. Я. В кн.: Антибиотики. М., 1959, с. 236. — Соболев В. Р. Анти тики тетрациклинового ряда. М., 1961. — Хроника ВОЗ, 1963, т. 17, № 10, с. 3 Böttiger L. E., Acta med. scand., 1955, v. 151, p. 343. — Helander S., Böttig L. E., Ibid., 1953, v. 147, p. 71.

Поступила 23/II 19

HYGIENIC SUGNIFICANCE OF THE USE OF TETRACYCLINES IN POULTRY FARMING

V. E. Shneiderman

The paper deals with certain problems connected with the feeding of poultry w high doses of tetracycline. The findings confirmed the ability of chlortetracycline (CT to accumulate in the organs and tissues of ducks due to its long-termu se. CTC proved be present in the organs and tissues on the 7th day after ceasing feeding. The boiling dusks' organs and tissues for a period of 90 minutes bid not produce complete inactivat of the traces of CTC present in the raw products. A definite relationship was establi between the relatively high retention of CTC in the organs and tissues and the amo of water present in the product after its boiling for one hour and a half. It is sugges that tissue proteins may have a protecting effect on CTC during the therm treatment.

УДК 616-006.4-02:664.8.035-0

ВЛИЯНИЕ НЕКОТОРЫХ ПИЩЕВЫХ КОНСЕРВАНТОВ НА РАЗВИТИЕ ЗЛОКАЧЕСТВЕННОГО ПРОЦЕССА У МЫШЕЙ

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В пищевой промышленности резко возросло применение разнообразных химических веществ. Возможность токсического и канцерогенного действия этих добавок в продуктах питания при их длительном попадании в человеческий организм недостаточно изучена.

Бисульфитом натрия обрабатывают фрукты, овощи и ягоды для предохранения их от плесени. Допустимая остаточная концентрация его до 300 мг на 1 кг. Бензойную кислоту используют в основном для обработки тех же продуктов и приготовляемых из них консервов с целью предупреждения брожения; ее концентрация не превышает 0,2% веса продукта. Оба консерванта применяют уже около полувека.

До недавнего времени бисульфит натрия и бензойную кислоту считали относительно безвредными, неканцерогенными (Kiechebusch и Lang; Hosino; Lockett и Natoli). Однако появился ряд работ, указывающих на отрицательное действие этих веществ на животный организм (Fitzhugh и соавторы; Morgan), в связи с чем в некоторых странах (Франция) применение бензойной кислоты запрещено, а вопрос о сернистых соединениях вынесен на обсуждение ВОЗ.

Сорбиновая кислота и низин — новые консерванты. Сорбиновая кислота, выделенная впервые из ягод рябины, оказалась очень эффективным консервантом. Она является полиненасыщенной жирной кислотой с 6 атомами углерода. По литературным дан

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ıом изучении.
возможного различ-
ıми тканей живого
нтибиотика парентe-
посмертные измене-
нтибиотиков с целью

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. т. 17, № 10, с. 369. —
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Поступила 23/II 1966 г.

ACYCLINES IN

feeding of poultry with
chlortetracycline (CTC)
termu se. CTC proved to
feeding. The boiling of
uce complete inactivation
tionship was established
tissues and the amount
a h. It is suggested
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ДК 616-006.4-02:664.8.035-092.9

ЕРВАНТОВ
ССА У МЫШЕЙ

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ным (Lang), добавление 5% сорбиновой кислоты к рациону крыс не вызывало никаких патологических изменений, а добавление 8% того же вещества (Deuel и соавторы) приводило к увеличению веса печени. Каких-либо указаний на канцерогенные свойства сорбиновой кислоты в литературе мы не обнаружили.

Антибиотик низин впервые был выделен из молочнокислых продуктов. Он является продуктом жизнедеятельности молочнокислых стрептококков, не имеет медицинского значения, но снижает сопротивляемость спор термостабильных бактерий к нагреванию, что позволяет при добавлении его в консервы снижать уровень стерилизации и тем самым повышать пищевую ценность консервов. Данные о канцерогенности низина отсутствуют.

При изучении токсичности бензойной кислоты в концентрациях, в которых это вещество содержится в продуктах питания человека, у мышей обнаружена наименьшая выживаемость и наименьший прирост веса тела, понижение детоксикационной способности печени, наибольшая степень выраженности отрицательного действия на организм животных при действии холода и пребывании в центрифуге (А. Д. Игнатьев).

Комбинированное действие бензойной кислоты и бисульфита натрия также влекло за собой резкое снижение выживаемости мышей, уменьшение потребления ими воды и корма, снижение веса тела и выносливости к физической нагрузке и голоданию. Отмечена также гипертрофия некоторых органов, особенно печени.

Сорбиновая кислота оказалась нетоксичной. Введение ее в рацион животных привело к более высоким, чем у контрольных животных, показателям выживаемости, увеличения веса, потребления корма, фагоцитарной активности и т. д.

Низин обладал наибольшей токсичностью, она проявилась и в смеси его с сорбиновой кислотой. Вместе с тем наблюдался и некоторый антагонизм действия сорбиновой кислоты и низина. В основном все перечисленные выше показатели больше приближались к норме, чем при введении одного низина, возможно, за счет благоприятного действия сорбиновой кислоты.

Во всех опытах, где мыши получали один низин или низин вместе с сорбиновой кислотой, обнаружена бо́льшая прибавка их веса. В одной серии опытов установлено также ожирение — вес тела превышал 40 г у 44% подопытных мышей.

Встречались случаи ожирения и среди животных, получавших бисульфит натрия совместно с бензойной кислотой, причем оно было выражено преимущественно у самок (А. Д. Игнатьев).

В наших исследованиях использовано 365 мышей обоего пола. Они были разделены на 5 групп: четыре опытных и одна контрольная. Все животные получали одинаковый рацион, вначале из натуральных продуктов (по нормам АМН СССР), а затем из брикетированных кормов. Подопытным мышам добавляли в корм изучаемые консерванты: 0,2% бензойной кислоты, 0,01% низина, 0,2% сорбиновой кислоты или 0,4% бисульфита натрия (по весу рациона). Дозы консервантов соответствовали тем, которые вводятся в продукты питания человека. В специальных группах мышей мы изучали комбинированное действие смеси бисульфита натрия с бензойной кислотой и смеси сорбиновой кислоты с низином. Подопытные животные в течение 3 месяцев получали эти консерванты с пищей в количестве, указанном выше, после чего им и контрольным животным внутрибрюшинно перевивали асцитный рак Эрлиха.

Как известно, при введении в брюшную полость мышей 50—80 млн. опухолевых клеток асцитный рак Эрлиха развивается у 100% перевитых мышей, длительность их жизни после перевивки составляет 12—17 дней. Нашими предварительными наблюдениями установлено, что при введении меньшего числа опухолевых клеток (1,2—1,25 млн. клеток) опухоли развиваются у 70—100% мышей, длительность их жизни несколько больше (21—30 дней).

Для того чтобы более четко установить влияние указанных консервантов на развитие асцитного рака, мы пользовались именно таким методом перевивки. Перед опытом мышей взвешивали и измеряли объем их живота. В дальнейшем измерения проводились каждые 4 дня со дня трансплантации опухоли и до гибели животных. Длительность наблюдений за ними составляла 53—66 дней, по истечении этого срока всех выживших мышей забивали и вскрывали. У всех мышей (погибших и забитых) измеряли количество асцитической жидкости и отмечали степень ее геморрагичности.

Последнюю обозначали в единицах следующим образом: +слабо-розовая ок... 1 единица; красная — 2 единицы и темно-красная — 3 единицы. Полученные д... были обработаны статистически; показатель существенности (*P*) определял... сравнении показателей данной группы с контрольной группой.

Полученные данные представлены в табл. 1 и 2.

Как видно из табл. 1 и 2, опухоли чаще перевивались у мышей, п... лучавших бисульфит натрия и бензойную кислоту раздельно, и в м... шей степени при совместном введении обоих веществ. Наименьшая д... тельность жизни мышей после трансплантации опухоли была у мыш... получавших раздельно бисульфит натрия и бензойную кислоту; об...

Таблица

Результаты прививки асцитного рака Эрлиха мышам, получавшим консерванты

Консервант	Число мышей	Длительность наблюдений (в днях)	Число опухолей		P
			абс.	%	
Бисульфит натрия	97	53	61	62,9±6,5	<0,0
Бензойная кислота	90	53	62	60,8±5,1	<0,0
Бисульфит натрия + бензойная кислота	22	66	9	40,9±10,5	<0,0
Сорбиновая кислота	58	66	17	29,3±6,0	
Сорбиновая кислота + низин	49	66	15	30,6±6,1	
Контроль	49	66	16	32,6±6,5	

Таблица

Длительность жизни мышей с асцитным раком Эрлиха, средний объем асцитической жидкости и степень ее геморрагичности

Консервант	Начало гибели мышей (в днях)	Средний объем асцитической жидкости (в *мл*)		Степень геморрагич... (в единиц...
		$M \pm m$	P	
Бисульфит натрия	12	6,5±0,418	<0,002	2,7
Бензойная кислота	15	5,03±0,330	<0,001	2,5
Бисульфит натрия+бензойная кислота	24	3,4±0,476	0,001	2,5
Сорбиновая кислота	21	5,06±0,626	>5	2,6
Сорбиновая кислота + низин	21	4,09±0,446	>5	3,0
Контроль	24	5,03±0,86		2,5

: +слабо-розовая окраска
тиницы. Полученные данные
ности (*P*) определяли при
руппой.

евивались у мышей, по-
у раздельно, и в мень-
ществ. Наименьшая дли-
опухоли была у мышей,
зойную кислоту; объем

Таблица 1

получавшим консерванты

Число опухолей		*P*
абс.	%	
61	62,9±6,5	<0,001
62	60,8±5,1	<0,001
9	40,9±10,5	<0,01
1	29,3±6,0	
15	30,6±6,1	
16	32,6±6,5	

Таблица 2

иха, средний объем
рагичности

ем асцитической сти (в *мл*)		Степень геморрагии (в единицах)
	P	
	<0,002	2,7
	<0,001	2,5
	0,001	2,5
	>5	2,6
	>5	3,0
		2,5

асцитической жидкости был наибольшим у мышей, получавших бисульфит натрия. Процент развившихся опухолей у мышей, получавших сорбиновую кислоту отдельно или вместе с низином, не отличался от того, который наблюдался у контрольных мышей, не получавших дополнительно консервантов. Длительность жизни этих мышей после трансплантации была незначительно меньшей, чем контрольных, объем асцитической жидкости у мышей, получавших сорбиновую кислоту, был таким же, как и у контрольных, а у мышей, получавших сорбиновую кислоту и низин, незначительно меньшим. Степень геморрагичности асцитической жидкости в условных единицах была одинаковой во всех группах.

Таким образом, добавление к рациону бисульфита натрия и бензойной кислоты способствовало более интенсивному развитию асцитного рака Эрлиха.

Мы изучали также влияние веса тела на частоту развития асцитного рака Эрлиха при введении в рацион консервантов. Как указывалось выше, при введении в рацион подопытных мышей сорбиновой кислоты и низина, а в отдельных случаях и бисульфита натрия с бензойной кислотой отмечалась тенденция к резкому повышению веса тела животных. У 40% мышей вес тела оказался выше 40 г, а в отдельных случаях достигал 50—60 г.

По литературным данным, в случае ожирения мышей, вызываемого ауротиоглюкозой, наблюдается более интенсивное развитие злокачественных опухолей. Так, Wexler указывает, что спонтанные опухоли молочной железы возникали раньше и чаще у мышей линии А с ожирением, вызванным ауротиоглюкозой.

То же самое отмечается и в отношении развития спонтанной гепатомы у мышей-самцов линии C_3H.

В наших опытах 90 мышей получали в течение 8 месяцев либо смесь сорбиновой кислоты с низином, либо бисульфита натрия с бензойной кислотой в дозах, указанных выше. Ввиду того что ожирение наблюдалось в различном проценте случаев в зависимости от пола животного (чаще у самок), мы включали в опыт самцов и самок. Опыты ставили как на ожиревших мышах, так и на мышах обычного веса. Для этого мыши были разбиты на 9 групп, по 10 мышей в каждой группе (самцы и самки обычного веса, самцы и самки с повышенным весом, контрольная группа).

Опыт должен был дать ответ на вопрос, способствует ли введение в рацион консерванта более интенсивному развитию злокачественного процесса или же это влияние является вторичным как следствие ожирения, вызываемого данным консервантом.

Всем мышам внутрибрюшинно вводили 1,2—1,25 млн. клеток асцитного рака Эрлиха. Разницы в развитии опухоли у мышей различных групп нам не удалось наблюдать, тем не менее длительность жизни животных и объем асцитической жидкости у них были неодинаковыми. Так, длительность жизни мышей, получавших смесь бисульфита натрия с бензойной кислотой, была в среднем несколько меньше после трансплантации опухоли (11—13 дней), а объем асцитической жидкости — несколько бóльшим (9—11 мл) по сравнению с остальными группами (у контрольных мышей соответственно 15 дней и 7—8 мл). Разница была статистически не достоверна, однако проявившаяся тенденция характеризовала бóльшую злокачественность асцитного рака Эрлиха у мышей, получавших бисульфит натрия вместе с бензойной кислотой. Подобное влияние, очевидно, не связано со способностью этих консервантов вызывать ожирение.

Начало гибели мышей, характеризующее интенсивность развития опухоли, объем асцитической жидкости и степень ее геморрагичности показаны в табл. 2.

Выводы

1. При трансплантации асцитного рака Эрлиха мышам, получавш с кормом уже ранее применявшиеся консерванты — бисульфит нат и бензойную кислоту, число развившихся опухолей было наименьш Длительность жизни этих мышей была наименьшей, а объем асцит ской жидкости — наибольшим. Это свидетельствует о большей злока ственности развившихся у них опухолей по сравнению с контрольны мышами.

2. Процент развившихся опухолей у мышей, получавших сорби вую кислоту и низин, а также степень ее злокачественности были та ми же, как и у контрольных мышей.

ЛИТЕРАТУРА

Игнатьев А. Д. Вопр. пит., 1965, № 3, с. 61. — Deuel H. J., Alfin-Slater R., Weie C. S. et al., Food. Res., 1954, v. 19, p. 1. — Fitzhugh O. G., Knudsen L. F., Nelson A. A., J. Pharmacol. exp. Ther., 1946, v. 86, p. 37. — Hosino. H. J. Hartwell. Survey of Compounds Which Have bun Tested for Carcinogenic activi Washington, 1951, p. 54. — Kieckebusch W., Lang K., Arzneimittel.-Forsch., 19 Bd 10, S. 1001. — Lang K., Ibid., S. 997. — Lockett M. F., Natoff I. L., J. Phar Pharmacol., 1960, v. 12, p. 488. — Morgan A. F., Am. J. publ. Hlth., 1935, v. 25, p. 328. Waxter S., Tabar P., Melcher L., Cancer Res., 1953, v. 13, p. 276. — Waxler S. J. nat. Cancer. Inst., 1954, v. 14, p. 1253. — Idem, Stanford med. Bull., 1957, v. 15, p. 26. Idem, Am. J. clin. Nutr., 1960, v. 8, p. 760.

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EFFECT OF CERTAIN FOOD CONSERVANTS ON THE DEVELOPMENT OF MALIGNANT PROCESSES IN MICE

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In tests performed over 365 mongrel mice of both sex was studied the effect of certain food conservants (sodium bisulfite, benzoic acid, sorbis acid, nizine) on the development of ascetic Erlich cancer. Those mice, that received sodium bisulfite and benzoic acid, presented a higher percentage of developed tumours, a shorter life length and a larger amount of ascetic fluid than the mice, receiving sorbic acid and nizine as well as the control animals. The fattening caused by the conservants had no effect on the development of the malignant processes.

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О ТОКСИЧЕСКОМ ДЕЙСТВИИ СЕВИНА НА ЖИВОТНЫЙ ОРГАНИЗМ

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В ближайшее время предполагается широко использовать в борьбе с вредителями продовольственных культур инсектициды — производные карбаминовой кислоты. Наиболее перспективным препаратом из этой группы является севин (1-нафтил-N-метилкарбамат) — кристаллический порошок кремового цвета, стабильный при хранении, с температурой плавления 142°, слабо растворимый в воде (0,03%) и умеренно в органических растворителях (до 40%).

Для использования в сельскохозяйственном производстве севин выпускают в виде смачивающихся порошков (30—80%), дустов (5—50%), концентрированных суспензий и гранул с различным содержанием активнодействующего вещества. Севин представляет собой инсекто-акари-

New Experiments on the Stabilization of Juices, Wines and Beverages with Potass: Sorbate

By Paolo Rovesti and Maria Galli

In a previous paper, written jointly with Dr. Pacetti, we reported on sorbic acid as stabilizer of juices, wines and beverages. After this paper, published in this Journal (*), much further literature has been added to the existing on the stabilization of food products by means of sorbic acid and the sorbates. Besides, several countries have confirmed their authorization to use such a prod uct as preservative, among them Sweden, Germany, the United States, and recently for wines, also France. Also Japan has officially approved this chemical preservation additive, after long experiments which confirmed its harmlessness.

While there have been various publications on the harmlessness of these products for the body, sufficient experimentation has not been made on the practical level in Italy by consumers, who are still too unfamiliar with these products.

One of the principal conditions, for example, which must be observed in stabi ization with sorbates is that which relates to the pH, for, as we had noted, at a low pH not below 3 the antifermentative and antimycotic action of sorbic acid is highest, while it decreases with increasing pH until it disappears beyond pH 7 The pH values normally considered for food substances are generally around 6, while for beverages much better conditions are obtained at lower pH values. It must be remembered that the fungistatic and antienzymatic action of sorbic acid depends on the undissociated part of the acid, which acts as preservative. In fact, the percentages of undissociated acid at the various pH values are the following:

pH 3	98%	undissociated	sorbic	acid
pH 4	86%	"	"	"
pH 5	37%	"	"	"
pH 6	6%	"	"	"
pH 7	0.06%	"	"	"

Another favorable condition of use is to start with potassium sorbate, which

is much more soluble in water, whereas sorbic acid is much less so, for the stab ilization of food products in the aqueous phase.

The reproach that sorbic acid reduces the aroma and flavor of the products ha repeatedly been contested in the literature, and on the basis of the experiments we have made we have not found it justified. On the contrary, especially with regard to wines, we noted an improvement of the aroma, and the same may be said of fruit juices.

Also the reproach about a possible reduction of the activity of sorbic acid a of the sorbates upon pasteurization proved unfounded after the numerous tests made. The preservative power remains unchanged even after prolonged pasteurization, as long as the pH of the juices and beverages is definitely acid.

However, these preservatives cannot, as some producers had hoped, prevent the gradual browning of the juices, or the chemico-enzymatic oxidations on aroma substances, carotenoids, polyphenols, and tannic substances.

We have examined also the action of sorbic acid on the pectins and have found that it does not in any way modify these important constituents of juices and beverages.

In order to complete our previous research and to report on the average results obtained on a much larger number of samples than that of our first paper, we have taken into consideration other juices (besides those tested before) and other sweet wines. The results of these experiments are reported in the tables.

The juices, sweet wines and juice beverages were prepared under highest conditions of hygiene and cleanliness. The fruits were washed and asepticized by washing with water, and the juices obtained were kept in sterile containers. As soon as they were prepared, potassium sorbate was added; they were temperature controlled at 30° and examined after one month. After this period, for the unchanged juices it could be considered that, with the hermetic sealing carried out, the stabilization was permanent.

The potassium sorbate was previously dssolved in a mother solution 2:10 of water, and for the various tests the solutions were added to the juices and wines tried.

We have marked with + the occurrence of fermentation and alteration of the juices, wines and beverages (sometimes with mould appearing on the surface), and with - their stability. The containers, all previous asepticized, were closed with hermetically sealing sterile stoppers. The products were not previously or subsequently pasteurized, but simply pressure-filtered through Seitz, so as to have clear juices and solutions.

As to the wines, they were tested at different acidity, since the pH, as stat before, considerably affects the dissociation of the sorbic acid.

The sweet wines marked A had 15% sugar, 0.6% acidity, and 5% alcohol by weight; the sweet wines marked B had 15% sugar, 0.9% acidity, and 6% alcohol by weight. A and B contained 250 mg/liter of free SO_2.

Prepasteurization of juices greatly reduces the stabilization doses. We found a reduction by half (0.5 g per mille of SK necessary), but it must be assumed that under industrial conditions of perfect hygiene and aseptic handling this does can be further reduced.

No disorder was caused by ingestion of even massive quantities of the stabilized products.

Summarizing Conclusions

As can be seen from the tables and from the foregoing, the following may be noted:

1) With respect to the fruit juices, prepared in the laboratory with all aseptic precautions, stabilization required a dose of at least 1 g per liter of juice, which is rahter higher than many data reported in the literature.

2) If the juices are previously pasteurized, preservation is obtained with half the dose. And since industrial plants always apply flash pasteurization or prior scalding of the fruit (before passing them through colanders or filters), under optimal hygienic conditions industrial stabilizations can be obtained with smaller doses than those found by us for prepasteurized juices.

Fruit juices	Test	SK 0.05%	0.1%	0.15%	0.2%	0.25%	0.3%	0.4%
Apple	+	+	-	-	-	-	-	-
Orange	+	+	-	-	-	-	-	-
Tomato	+	+	-	-	-	-	-	-
Lemon	+	+	-	-	-	-	-	-
Aprico	+	+	-	-	-	-	-	-
Peach	+	+	-	-	-	-	-	-
Pear	+	+	-	-	-	-	-	-

3) The sweet wines containing 250 mg of SO_2 per liter (with 15% sugar, 6% by weight of alcohol and with 0.9% acidity) are preserved already with 0.15 g of SK per liter. This proves the effect of the acidity on the dissociation of the sorbate and hence on the stabilization.

4) The sweet wines containing no SO_2 (with 15% sugar, 6% by weight of alcohol and 0.9% acidity) are preserved at obviously higher doses than those treated with SO_2. The wines with higher acidity (0.9%) are stabilized with 400 mg/liter of SK, while the wines of lower acidity (0.6%) require at least 500 mg.

Wines	Test	Sk 0.15	0.2	0.25	0.3	0.4	0.5	1 per mille
Sweet white A	+	-	-	-	-	-	-	-
Sweet white B	+	+	-	-	-	-	-	-
Sweet red A	+	-	-	-	-	-	-	-
Sweet red B	+	+	-	-	-	-	-	-
Sweet white C	+	+	+	+	+	-	-	-
Sweet white D	+	+	+	+	+	+	-	-
Sweet red C	+	+	+	+	+	-	-	-
Sweet red D	+	+	+	+	+	+	-	-

5) The juice beverages are stabilized with 600 mg per liter also at high doses of juice (20 and 25%). With smaller doses of juice for lemonades (12 and 15%) stabilization is obtained also with 500 mg of SK per liter.

Juice beverages	Test	SK 0.4	0.5	0.6	0.7	0.8	0.9	1 per mille
Orangeade I (15%)	+	+	-	-	-	-	-	-
Orangeade II (20%)	+	+	+	-	-	-	-	-
Orangeade III (25%)	+	+	-	-	-	-	-	-
Lemonade II (12%)	+	-	-	-	-	-	-	-

6) There has never been trouble with even massive ingestion of the products stabilized with SK.

- - -

We close with thanks to the firm Emelfa of Milan, which made available free of charge the potassium sorbate (SK) used in this research.

Milan, Istituto di Ricerche sui Derivati Vegetali, April 1962.

Bibliography

To the bibliography already published by us in No. 11 of this Journal should be added the following papers:

E. Kielhöfer, Biologic Stabilization of Low-alcohol Wines by Sorbic Acid, in "Weinblatt" 1960, No. 45.

K. Lang, The Tolerance of Sorbic Acid, in "Arzneimittel-Forschung" 1960, 997.

D. H. Cremer, W. Tolckmitt, J. Wenderhold, Contribution to the Physiology of Sorbic Acid, in "Klin, Wochenschr." 1959, 304.

E. Lück, Sorbic Acid as Preservative for Sweets, in "Kakao-Zucker" 1961, 12.

E. Lück, Sorbic acid as a Preservative, in "Soft Drinks Trade Journal" 1960, October.

(*) No. 11 (March-April 1961), p. 101.

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Nuove esperienze sulla stabilizzazione di succhi, vini e bevande con sorbato potassico

PAOLO ROVESTI - MARIA GALLI

In una precedente nota, eseguita in collaborazione con la dott. Pacetti, è stato riferito circa l'acido sorbico come stabilizzante di succhi, vini e bevande. Dopo questa nota, comparsa su questa Rivista (*), molta altra letteratura è venuta ad aggiungersi a quella già esistente sulla stabilizzazione di prodotti alimentari a mezzo dell'acido sorbico e dei sorbati. Inoltre, alcuni Paesi hanno confermato la loro autorizzazione ad impiegare tale prodotto come conservativo e tra essi la Svezia, la Germania, gli Stati Uniti e recentemente, per i vini, anche la Francia. Anche il Giappone ha ufficialmente permesso questo additivo chimico di conservazione, dopo lunghe esperienze che ne hanno confermato l'innocuità.

Mentre diversi lavori sono stati pubblicati sulla innocuità per l'organismo di questi prodotti, sul piano pratico non sono state fatte sufficienti sperimentazioni in Italia da parte di consumatori, che ancora conoscono troppo poco questi prodotti.

Una delle condizioni principali ad esempio che occorre osservare nella stabilizzazione con sorbati è quella che si riferisce al pH in quanto, come avevamo ricordato, a pH basso non inferiore a 3 l'azione antifermentativa ed antimicotica dell'acido sorbico è massima, mentre decresce con l'innalzarsi del pH sino ad annullarsi oltre pH 7. Generalmente i pH normalmente considerati per le sostanze alimentari stanno intorno a 6, mentre per le bevande si hanno condizioni molto migliori ai pH inferiori. Si deve ricordare che l'azione fungistatica ed antienzimatica dell'acido sorbico dipende dalla parte non dissociata dell'acido, che agisce come preservante. Si hanno infatti i seguenti valori di percentuali di acido non dissociato ai diversi pH:

pH 3	98%	acido sorbico non dissociato
pH 4	86%	» » » »
pH 5	37%	» » » »
pH 6	6%	» » » »
pH 7	0,06%	» » » »

Altra condizione favorevole di impiego è quella di partire dal sorbato di potassio, molto più solubile in acqua, invece che dall'acido sorbico che lo è molto meno, ai fini della stabilizzazione di prodotti alimentari in fase acquosa.

Gli appunti fatti all'acido sorbico di minimizzare l'aroma ed il gusto dei prodotti è già stato più volte contestato dalla letteratura e noi stessi, dalle esperienze eseguite, non li abbiamo trovati affatto fondati. Viceversa, specialmente per quanto riguarda i vini, abbiamo notato un miglioramento dell'aroma e così dicasi per i succhi di frutta.

Anche gli appunti ipotetici su una possibile minorazione di attività dell'acido sorbico e dei sorbati con la pastorizzazione sono risultati, dalle numerose prove eseguite, infondati. Il potere conservante si mantiene inalterato anche per prolungate pastorizzazioni, purché il pH dei succhi e delle bevande sia nettamente acido.

Viceversa, questi prodotti preservanti non possono, come qualche produttore si aspettava, impedire le reazioni di imbrunimento nel tempo dei succhi, né le ossidazioni di ordine chimico-enzimatico sugli aromi, sui carotenoidi, sui polifenoli e sulle sostanze tanniche.

Anche l'azione dell'acido sorbico sulle pectine è stata da noi esaminata ed è stato trovato che esso non modifica in alcun modo questi così importanti costituenti di succhi e bevande.

Onde completare le nostre ricerche precedenti e riferire sui risultati medi ottenuti su un numero di campioni ben maggiore di quello della nostra prima nota, abbiamo preso in considerazione altri succhi (oltre a quelli precedentemente saggiati) ed altri vini dolci. Nelle tabelle riportate sono esposti i risultati di queste esperienze.

I succhi, i vini dolci e le bevande a succo venivano preparati nelle massime condizioni di igiene e pulizia. Venivano previamente lavate e asettizzate con lavaggi acquosi le frutta di partenza ed i succhi ottenuti venivano conservati in recipienti sterili. Appena preparati venivano addizionati di sorbato potassico, termostatati a 30° ed esaminati dopo un mese. Dopo questo periodo la stabilizzazione poteva, per i succhi mantenutisi invariati, considerarsi permanente, alla chiusura ermetica praticata.

Il sorbato potassico veniva preventivamente disciolto in una soluzione madre 2:10 di acqua e le soluzioni venivano aggiunte per le diverse prove ai succhi ed ai vini saggiati.

Abbiamo indicato con + l'avvenuta fermentazione ed alterazione dei succhi, vini e bevande (talora con

(*) N. 11 (marzo-aprile 1961), pag. 101.

comparsa di muffe in superficie) e con — la stabilità. I recipienti, tutti preventivamente asettizzati, venivano chiusi con tappi sterili a tenuta ermetica. I prodotti non venivano pastorizzati preventivamente o successivamente, ma semplicemente filtrati a pressione Seitz, in modo da avere succhi e soluzioni limpide.

Per quanto riguarda i vini, essi sono stati sperimentati con diversa acidità, in quanto il pH, come abbiamo detto precedentemente, incide notevolmente sulla dissociazione dell'acido sorbico.

I vini dolci indicati con A avevano il 15% di zuccheri, 0,6% di acidità e 5% di alcool peso; i vini dolci indicati con B avevano il 15% di zuccheri, 0,9% di acidità e 6% di alcool peso. In A e B erano contenuti 250 mgr/litro di SO_2 libera.

Viceversa i vini dolci indicati con C e D avevano la stessa composizione di A e B (C con 0,6% di acidità e D con 0,9%), ma non contenevano SO_2.

Le prepastorizzazioni di succhi riducono notevolmente le dosi di stabilizzazione. Noi abbiamo trovato una riduzione della metà (0,5 gr. per mille di SK necessario). ma si deve presumere che in condizioni industriali di perfetta igienicità e cure asettiche questa dose si potrà ulteriormente ridurre.

Nessun disturbo è stato provocato da ingestioni anche massive dei prodotti stabilizzati.

Conclusioni riassuntive

Come si può dedurre dalle tabelle e da quanto precede, si può notare quanto segue:

1) Per quanto riguarda i succhi di frutta, preparati in laboratorio con tutte le precauzioni asettiche di contatto, è occorsa per la stabilizzazione una dose di almeno 1 gr. per litro di succo, assai superiore a molti dati riportati dalla letteratura.

2) Se i succhi vengono preventivamente pastorizzati, si consegue una conservazione con metà dose. E poiché industrialmente si operano sempre pastorizzazioni-lampo o scottature preventive delle frutta (prima dei passaggi in passatrici o filtri), si possono, a condizioni igieniche ottimali, ottenere stabilizzazioni industriali anche con dosi minori di quelle trovate da noi per succhi prepastorizzati.

Succhi di frutta	Test	SK 0,05%	SK 0,1%	SK 0,15%	SK 0,2%	SK 0,25%	SK 0,3%	SK 0,4%
Mela	+	+	—	—	—	—	—	—
Arancio	+	+	—	—	—	—	—	—
Pomodoro	+	+	—	—	—	—	—	—
Limone	+	+	—	—	—	—	—	—
Albicocca	+	+	—	—	—	—	—	—
Pesca	+	+	—	—	—	—	—	—
[illegible]	+	+	—	—	—	—	—	—

3) I vini dolci contenenti 250 mgr. di SO_2 per litro (con il 15% di zuccheri, il 6% peso di alcool e con il 0,9% di acidità) si conservano già con 0,15 gr. di SK per litro. Ciò comprova l'incidenza dell'acidità sulla dissociazione del sorbato e quindi sulla stabilizzazione.

4) I vini dolci non contenenti SO_2 (con il 15% di zuccheri, 6% in peso di alcool e 0,9% di acidità) si conservano a dosi ovviamente superiori rispetto a quelli trattati con SO_2. Si ha stabilizzazione per i vini con maggiore acidità (0,9%) con 400 mgr/litro di SK, mentre per i vini con minore acidità (0,6%) occorrono almeno 500 mgr.

Vini	Test	SK 0,15‰	SK 0,2‰	SK 0,25‰	SK 0,3‰	SK 0,4‰	SK 0,5‰	SK 1‰
Bianco dolce A	+	—	—	—	—	—	—	—
Bianco dolce B	+	+	—	—	—	—	—	—
Rosso dolce A	+	—	—	—	—	—	—	—
Rosso dolce B	+	+	—	—	—	—	—	—
Bianco dolce C	+	+	+	+	+	—	—	—
Bianco dolce D	+	+	+	+	+	+	—	—
Rosso dolce C	+	+	+	+	+	—	—	—
Rosso dolce D	+	+	+	+	+	+	—	—

5) Le bevande a succo vengono stabilizzate con 600 mgr. per litro anche a dosi forti di succo (20 e 25%). Con dosi minori di succo per le limonate (12 e 15%) si ha stabilizzazione anche con 500 mgr. di SK/litro.

Bevande a succo	Test	SK 0,4‰	SK 0,5‰	SK 0,6‰	SK 0,7‰	SK 0,8‰	SK 0,9‰	SK 1‰
Aranciata I (15%)	+	+	—	—	—	—	—	—
Aranciata II (20%)	+	+	+	—	—	—	—	—
Aranciata III (25%)	+	+	+	—	—	—	—	—
Limonata I (15%)	+	+	—	—	—	—	—	—
Limonata II (12%)	+	—	—	—	—	—	—	—

6) Non si sono mai avuti inconvenienti in ingestioni anche massive dei prodotti stabilizzati con SK.

* * *

Ringraziamo alla fine della presente nota la Ditta Emelfa di Milano, che ci ha messo gratuitamente a disposizione il sorbato di potassio (SK) impiegato nelle presenti ricerche.

Milano, Istituto di Ricerche sui Derivati Vegetali, aprile 1962.

BIBLIOGRAFIA

Alla bibliografia già da noi pubblicata nel n. 11 della presente Rivista sono da aggiungere i seguenti lavori:

Kielhöfer E., *Die biologische Stabilisierung der alkoholarmen Weine durch Sorbinsäure*, in « Weinblatt », 1960, n. 45.

Lang K., *Die Verträglichkeit der Sorbinsäure*, in « Arzneimittel-Forschung », 1960, 997.

Cremer D. H., Tolckmitt W., Wenderhold J., *Beitrag z. Physiologie der Sorbinsäure*, in « Klin. Wochenschr. », 1959, 304.

Lück E., *Sorbinsäure als Konservierungsmittel f. Süssaren*, in « Kakao-Zucker », 1961, 12.

Lück E., *Sorbic acid as a Preservative*, in « Soft Drinks Trade Journal », 1960, October.

Kagaku Kogyo Zasshi (Journal of the Chemical Society of Japan, Industrial Chemistry Section), February 1967, pp. 121-125.

The Toxicity of Food Additives and Problems Involved.

By Kazuyoshi Aiso (Doctor of Medicine, Professor at the University of Chiba).

1. Introduction

In these days when as many as 400 different food additives are used, there may be many people who wonder whether food is safe and some people may even believe that it is not safe at all.

Particularly in recent years, there has been an increasingknowledge of cancer-producing substances and latent chronic toxicity and there has been a mounting doubt as to the safety of processed foodstuff.

Such a trend exists not only in Japan but also in European countries and there is a trend toward more stringent regulations of food additives in many countries. The international research and co-operative organs in the field of food additives include the World Health Organization (WHO), the Food and Agriculture Organization (FAO) and UICC (Union of International Cancer Committees) and so forth. The activity of these organizations relative to the evaluation of the safety of food additives has recently been markedly increased.

As for the reasons why such doubts have come into existence (let us limit our thinking to the situation in our own country on a tentative basis), there is an excessive use of food additives as represented by food pigments, etc. on one hand. On the other hand, there is a growing mistrust in the law-abiding spirit of the people in the food industry. Behind this, there is a prevailing lack of knowledge regarding the toxicity of additives.

What is the actual situation? It is indeed regretful that I do not have the courage to state categorically that all doubts about the food additives are unfounded. Nevertheless, the actual situation is not so serious as it is feared to be. If an evaluation is made on a scientific manner in a calm state of mind, it can be stated that some degree of safety is currently maintained.

Most of the 400 food additives which are being used are such substances which are not harmful to human health, judging from their chemical properties. However, the present situation is such that some of these additives are believed to be somewhat doubtful. These doubts are directed toward artificial coloring agents, food preservatives, bleaching agents and artificial sweetening agents, etc.

Let us discuss the problems of toxicity in view of the current toxicity testing methods relative to food coloring agents and food preservatives. Since there are many medical and technical terms to be used in the evaluation of the toxicity of chemical substances, reference should be made to the directions concerning food additives and to the deliberation standards of the Food Hygiene Deliberation Council at the time when the standards were set (Refer to the Study of Food Hygiene, 15, No. 10, 3, 1965).

2. Food Pigments (Coloring Agents)

2.1. Present Status of Food Pigments (Coloring Agents).

In the olden days when food coloring agents were selected from among the synthetic coloring agents without carrying out a chronic toxicity test as in these days, it was natural that substances having a high degree of toxicity were mixed. During the course of the administrative changes relative to food additives in our country, picric acid, dinitrocresol, methyl violet, Auramine, Rhodamine, etc. have been banned as pigments (coloring agents) possibly causing acute toxicity.

Since there was a danger of arsen, lead and other harmful substances being mixed in the manufacturing process, an "Announcement relative to the Sealing of Coloring Agents" was made public in 1941 and the sale of a manufactured food was made contingent upon the obtaining of a governmental approval from the National Hygiene Testing Institute. In 1948, 24 coloring agents of the tar system and a part of lead compounds were designated on the basis of the Rules relative to the Enforcement of the Law pertaining to Food Hygiene.

The coloring agents of the tar system have satisfactory coloring properties

and offer a wide variety of color choices. They made it possible to produce the products of high purity at low cost in mass production. Therefore, most of the coloring agents used currently are of the tar system.

However, even among the 24 coloring agents of the tar system without the coloring agents of high toxicity, there are some problems when they are examined as to their toxicity on the basis of the current scientific level. Twelve (12) of the 24 coloring agents of the tar system are pigments of the azo system. The azo pigments are the substances in which our country achieved a great result in the history of a study of chemical substances leading to the growth of cancer. In 1932, Messrs. Takaoki Sasaki and Tomizo Voshida succeeded in producing liver cancer in rats by using orthoamine azo toluol for the first time. In 1937, Mr. Ryojun Kinoshita, discovered the fact that dimethyl aminoazobenzene was a powerful cancerous pigment producing liver cancer. This substance is a coloring agent of the tar system which was called butter yellow and is currently used throughout the world as a substance which is indispensable in the study of cancer production.

What is the relationship between this substance and the food coloring agents of the azo system? An examination of this problem in a long-term feeding test wherein two-year feeding was examined shows that some were completely safe from the standpoint of cancer production but that others were doubtful as will be described in detail later. Because of this, two coloring agents including Red No. 1 and Red No. 101 were removed from the designated list in 1966.

There are some fat-soluble tar pigments. Amongst these, there are some which are highly accumulative in the living cells of a high-class animal and have a high degree of toxicity by bringing about strong degeneration and even death in the liver cells. Red No. 3 is the case in point. On the coloring agents of the tar system, the committees of WHO, FAO and UICC, etc. have carried out energetic studies on the re-evaluation of their safety. Based on such re-evaluations, the various countries in the world have reduced the number of (approved) coloring agents belonging to the tar system.

In our country, measures were taken in 1967 (in January of this year) to

ban Red Nos. 4 and 5, Orange Nos.1 and 2, and Yellow No. 1, 2 and 3. Accordingly, the total number of the coloring agents belonging to the tar system has been drastically reduced from 24 to 15. Even among the 15 that are left, 12 coloring agents are still being examined. Then, what kind of toxicity has been discovered in these coloring agents of the tar system which have been found to be toxic?

2.2. Ponceau 3R and Sunset Yellow FCF

Ponceau 3R (Red No. 1) and Sunset Yellow (Yellow No. 5) are azo pigments having the same skeleton in structure but they show entirely different kinds of toxicity. When rats were fed with 0.5 per cent, one per cent and two per cent of these coloring agents, for instance, it was found that the animals ate the feed containing these coloring agents to a satisfactory degree. A comparison of these rats in terms of their growth shows the fact that there is no sign of growth inhibition as the curve representing the growth of their weight was the same as the curve representing the growth of these rats in the control group who were given the feed without such an addition for each concentration.

On the other hand, there is clearly observed the sign of a growth inhibition in the group of rats which were fed two per cent of Ponceau 3R and even the group which were fed one per cent showed a rate of growth which was inferior to that of the control group. A disection of the animals which had completed the two-year feeding test revealed the fact that, both by naked-eye observation and by the patholgical observations, the degeneration of substantial cells and the precipitation of fat were found in the main organs including the livers, etc. Moreover, no abnormal multiplication of liver cells and no signs of ulceration, such as liver cancer etc. were discovered, with the state being similar to those of the rats in the group which was given no additions.

In the group of Ponceau 3R, on the other hand, degenerated images were found in the liver cells, with the effect of the coloring agents being clearly observed. Even though the said degeneration is not of such a high degree, there were observed abnormal multiplications at a number of places in the liver

structure, with the growth of swelling being observed. In some cases, the deterioration was such that the situation could well be termed a liver cancer (reference should be made to Figure 1).

In terms of number, in 50 percent of the cases where more than 0.5 per cent was added, liver inflammation was proved. These data indicate the fact that Poceau 3R has a cancer-producing nature, even though to a small extent.

Sunset Yellow is one of the three coloring agents which were branded to be those food coloring agents having a high degree of safety by the committee of WHO and FAO. The present authors were also able to determine their safety without a shadow of doubt. Red No. 101 has been proved to have a weak cancer-producing property like Red No. 1. On this basis, its designation was cancelled in 1966.

2.3. Fat-soluble Tarry Pigments (Coloring Agents)

Since Oil Red XO (Red No. 5) is fat-soluble, it has its own special uses. When it was examined, however, its toxicity was unexpectedly high. When it was added in the range between one and two per cent, the rats died within a period of three to four months. When from 0.5 to 0.25 per cent was fed, 70 per cent of the rats died within a period of seven months. Even when only 0.1 per cent was fed, 60 per cent of the animals died. The cause of their death was mostly the necrose of the liver and fiberization takes place after the destruction of the cells, thereby producing the shape of small leaves (reference should be made to the photograph). When fat-coloring is carried out, the fat degeneration of a strong centricity was observed.

(Insert Figure 1 on p. 122. Structure of the liver in a rat which was developed after feeding Ponceau 3R.)

(Insert Figure 2 on p. 123. Structure of the liver when a rat was fed a feed containing 0.1 per cent of oil red (Red No. 5) for a period of 12 weeks, observe small pseudo-leaves which have been fiberized in small leaves.)

In order to carry out a long-term observation by preventing the early death of the animals, the authors gave the animals both the food with such an addition and the food without such an addition on an intermittent basis. After

feeding food containing from 0.1 to 0.25 per cent of an additive for a prescribed period of time, they were given food without any additive. This process was repeated for a period of one year and six months.

Even though the total results have not as yet been gathered and complied, it can be stated on the basis of the results which have been accumulated thus far that, although the status of the illness changes, those which develop the hardening and degeneration of the liver were rare. H wever, the abnormal multiplication of liver cells or the multiplication of tumorous growths was observed, thereby showing a sign of cancer production.

According to literature, when less than 0.02 per cent of this coloring agent is fed, no marked degree of toxicity is observed nor is the development of liver tumorous growth observed. It is believed that the cancer-producing character of the same can be proved by intermittently feeding the animals with feed of comparatively high concentration by the method of the present authors.

If toxicity is shown when the amount of the addition is 0.1 per cent, it can be stated that the said coloring agent has an accumulative character and its safety is not certain. Oil yellow AB (Yellow No. 2) and Oil Yellow OB (Yellow No. 3), etc. show this kind of toxicity. These two are the azo compounds of naphthyl amine and their cancer-producing properties were suspected since olden days. The fact that these three coloring agents were removed from the designated list in January of this year was a natural consequence.

3. Rot-preservatives

Even though the functional mechanism is different, the preservatives have common cell toxicity (cytotoxicity) in the sense that they have the capacity of preventing the multiplication of putrifying micro-organisms. Accordingly, they ought to be such chemical substances as ill have toxicity. In this sense, they should be discussed more stringently in terms of their safety than in the case of other additives.

As has already been understood, however there are such substances which, while showing an inhibitive action without fail on rottening micro-organisms, ultimately show an extremely mild effect on the cells and structures of the

living body which takes the same. As a preservative, such a substance is selected.

A number of conditions will have to be met in order for a substance to be harmful to micro-organisms but is harmless to the living body: First of all, (1) it has to be metabolized completely in the living body and converted into a harmless final product to be exhausted out of the body. (2) It should have no accumulative effect and the substance which has been absorbed has to be entirely exhausted through urine while it is converted into a substance of lower toxicity. (3) Even in the case of a substance having toxicity, its effect should be obtained when five ppm of it is added. It should not remain in the food for a long period of time and it should not be absorbed from the intestinal tubes but be exhausted together with the stool. If one of the above conditions is met, it is possible for us to permit the existence of a preservative having a high degree of safety by introducing the concept of a "permissible amount."

In this connection, it should be mentioned that the international standards for the evaluation of the toxicity of antimicrobials are quite stringent and those substances which have passed the examinations of the WHO and FAO special committees are indeed rare and small in number.

In our country, the use of food preservatives has been quite important in view of the comparatively small food resources, the dietary customs and habits of the people, the climatic conditions and the level of national income, etc. Thus, there is a possibility of food preservatives having been used too excessively. Below, the extent of the toxicity of the legal food preservatives in our country will be described.

3.1. Salicylic Acid

Salicylic acid and salicylate are taken through the mouth and are easily absorbed through the intestinal tubes. The acute toxicity LD_{50} which can be taken by a rat through feeding by mouth is in the range between 1,500 and 2,000 milligrams per kilogram. Thus, its toxicity does not deem to be too high. Because of its accumulative nature, more than 50 per cent die when 500 milligrams per kilogram is continuously fed for a period of from four to 21 days.

When 600 milligrams per kilogram is given, 100 per cent die within a period of 10 days. When the rats are weak and young, a sign of growth inhibition is observed within a period of six weeks when 0.5 per cent is added to the feed.

As a harmful effect upon blood, when 100 milligrams of this is fed to a rat per day, a severe low-thrombin blood case is developed.

In the case of a human being, salicylic acid has been used as a medicine for a long time since olden days. Its side effect is also well known. Toxicity which is created as a result of the excessive feeding of this substance is called salicylism. It not only develops a case of nerve center poisoning but it also brings about a destruction of acid-alkali equilibrium, metabolic hindrance, the organic degeneration of liver and kidney, etc. It is reported that the amount of the same required for the death of a human is in the range between 20 and 30 grams.

Strange as it may seem, however, there is no description (report?) as to long-term chronic toxicity tests in the case where a small amount has been fed for a long period of time. Because of this, no decisive evaluation can be made as a food preservative. According to the reports of the special committee of the WHO and FAO, it is not suitable as an additive.

In our country, the addition of the same in an amount of 0.25 grams per liter is permitted in Japanese sake (wine). A person who drinks one liter of Sake per day absorbs 250 milligrams of salicylic acid, which is equivalent to one one-hundredth of the amount required for a man to die. When the amount of the same required for a rat to die or 600 milligrams per kilogram is converted into the case of human beings, it will be 30 grams. If a safety co-efficient of 100 times is adopted,it will be 300 milligrams. Thus, the amount is indeed on the border line and offers a serious problem.

When it is taken along with alcohol, in particular, the toxicity of salicylic acid should be carefuly examined. We are hard put to understand why this problem has been left unsolved to this date.

3.2. Benzoic acid, sodium benzoate and para-oxy benzoic acid esters.

Benzoic acid is quickly absorbed into the living body. However, it is completely exhausted from the urine as harmless hippuric acid or united glucuronic acid. The mechanism of its absorption and exhaustion was proved in an experiment using benzoic acid which was labeled C14 in the case of a rat.

The low toxicity of this substance can be anticipated from this biochemical study. The LD_{50} of sodium benzoate is in the range between 2,700 and 2,000 milligrams per kilogram (rats, mice and dogs). In a chronic toxicity test, there is a report describing the feeding of a rat during its lifetime (approximately two years and a half) with the food to which this was added in an amount of 0.5 per cent and 1.0 per cent. Even in the case where one per cent of the same was added, there was no inhibition of the growth,with no difference being observed as compared with the control group. Disection showed no toxicity.

It is an interesting fact that salicylic acid and benzoic acid with OH added to the ortho position shows a difference in living metabolism and a difference in toxicity.

Regarding the para-oxy benzoic acid esters, detailed toxicity experiments on methyl, ethyl and propyl ester are reported in literature and LD_{50} is in the range between 2,000 and 3,700 milligrams per kilogram in the case of a mouse. In a chronic toxicity test, rats were fed for a period of 96 weeks with feed having two to eight per cent of the same added thereto. As a result, the growth of the bodily weight was inhibited in the case of propyl ester when eight per cent was added. Regarding three esters, the concentration of two per cent had no effect at all, thereby indicating the markedly low toxicity. In this manner,it is correct to assume that the safety level of benzoic acid, sodium benzoate and para-oxy benzoic acid esters is comparatively high as food preservatives.

3.3. Sorbic Acid and Propionic Acid Salts.

Sorbic acid is metabolized by (beta) oxidation in the same manner as capric acid to be ultimately converted into CO_2 and H_2O. In this case, the animals can utilize sorbic acid as a source of calories. In an experiment using rats, moreover, it was made clear that the same is not used as a metabolic antagonist

of essential fatty acids. In this manner, this is believed to be a substance of low toxicity from a biochemical viewpoint.

The LD_{50} of this item shows a value which is practically non-toxic at around 10,500 milligrams per kilogram. In a chronic experiment, there was observed no inhibition of growth during the period of 1,000 days when the animals were given feed containing five per cent of the same. Since no other toxic change was observed, no effect level will be five per cent (2,500 milligrams per kilogram).

Propionates are metabolized and utilized in the same route as regular fatty acids in a living body. In an experiment using mice, the propionic acid which was labelled at C^{14} was found as a forerunner of the bodily fat and C^{14} was further confirmed to be distributed to carbon dioxide, glucose, succinate and protein, etc.

In a long-term feeding experiment, there was no inhibition of the growth nor was there any rise in the death rate when the rats were given feed containing 3.75 per cent of the additive. From the standpoint of a pathological structural test, there was observed no toxicity. From these results, it can be stated that the propionates are also such preservatives having a high degree of safety.

3.4. Dehydroacetic acid

The acute toxicity of dehydroacetic acid was as follows: In the case of rats, the LD_{50} value was 1,000 milligrams per kilogram and in the case of a mouse, it was 1,270 milligrams per kilogram. In the case of a salt of sodium, the value was 570 milligrams per kilogram in the case of a rat and 1,050 milligrams per kilogram in the cas of a mouse. Therefore, it s toxicity is higher than the substances which have been discussed in the past. When 1.5 to 1.9 grams per kilogram is given to a mouse, it causes death. In the six-month continuous feeding of rats, no toxicity was observed when 0.1 grams per kilogram was given. In the case of 0.2 grams per kilogram, however, there is observed an inhibition of the growth. In the case of 0.3 grams per kilogram, further, the animals die in the neighborhood of two months.

In a chronic toxicity test, no toxicity was observed when rats were given

feed containing 0.1 per cent of the additive. In the case of a monkey, no abnormalcy was observed when 0.2 grams per kilogram was fed for a period of one year. The no-effect level will be 100 milligrams per kilogram. When this value is converted into the case of human beings and a safety co-efficient of 100 is used, the maximum permissible amount will be 50 milligrams of DHA per day. Therefore, within the currently approved food and use standards, its safety is guaranteed. Since there is not much leeway, however, no illegal use of the same in such foods as are not designated will be permitted. The LD_{50} values of preservatives which have been discussed and the no-effect levels will be shown in a table.

Table 1. Comparison of the toxicity of designated preservatives.

Preservative	Acute toxicity DL_{50} milligrams per kilogram.	Rat safety amount in a chronic toxicity test.
Salicylic acid	1,500-2,000 (rat)	There is no reliable data of a chronic toxicity test.
Benzoic acid Sodium benzoate	2,700 (rat)	One per cent in feed (500 milligrams per kilogram).
Methyl p-hydroxybenzoate	2,000 (mouse)	Two per cent in feed (1,000 milligrams per kilogram).
Propyl p-hydroxybenzoate	3,700 (mouse)	Two per cent in feed (1,000 milligrams per kilogram).
Propionate of sodium & calcium	2,600 (rat (propionic acid)	3.75 per cent in feed (2,000 milligrams per kilogram).
Sorbic acid	10,500 (rat)	5.0 per cent in feed (2,500 milligrams per kilogram).
Dehydroacetic acid	1,000 (rat) 1,270 (mouse)	0.1 per cent in feed (50 milligrams per kilogram).)

3.5. Furil furamide (Translator's Note: transliteration)

It is only in our country that some of the nitrofurane derivatives are used as food preservatices (antimicrobials). Therefore, the reference materials for its evaluation will have to be found among the research papers published in this country, Last year, furil furamide was freshly designated and the con-

ventional nitrofurazone and Z-furane were removed from the designated list. This may be due to the fact that its toxicity was lower than that of nitrofurane.

Different from the preservatives which have been described above, antibiotic nitrofurane is a full-fledged antibiotic substance and its antibiotic force is quite strong. In the case of furil furamide, it exerts its growth inhibitive effect upon many putrifying bacteria within five ppm. Therefore, it is possible to prevent the concentration of its addition to less than five ppm.

However, it cannot be called a substance with low toxicity. In terms of acute toxicity, its LD_{50} values is in the neighborhood of 570 milligrams per kilogram. Even when 0.1 per cent may be added to food, there is no inhibition of growth within a period of three months. When 0.2 per cent is added, they withstand feeding for a period of one year. Even when rats are fed at a concentration in the range between 0.01 and 0.1 per cent for a period of one to two years, there is no possibility of major organs being given a toxic change.

When less than 0.2 per cent is fed, the contraction of the testicles and the inhibition of the formation of sperms as in the case of nitrofurazone cannot be observed; however, a slight fattening of the liver is observed. It should be mentioned that this fattening of the liver is a balanced growh (expansion) of the substantive cells of the liver and is different from an abnormal multiplication. There is observed no trend toward swelling even after a long period of feeding. Professor Miyaji of Osaka University placed the no-effect level of this substance at 0.01 per cent. However, the present authors believe that (even though this is a result of the one-year feeding test) 0.02 per cent is a safe amount.

If 0.01 per cent is converted, the safe amount for rats will be five milligrams per kilogram. When this is converted to human beings and a safety coefficient of 100 times is adopted, the daily amount will be 2.5 milligrams of the concentration of an addition to food is set at one-two hundred thousandth, the permissible weight of additive food will be 500 grams. Within the range of approved foods at present, its safety is guaranteed. However, it should be

added that a study should be carried out in the future as to in what manner this chemical substance will be metabolized in the human body and what toxicity the metabolism product will have. It may be necessary to stipulate that a very small amount of such a full-fledged antibiotic substance should be added to a few specified kinds of food.

3.6. Antibiotic Substances

Whether it is correct to add an antibiotic substance to food for the purpose of maintaining freshness or not has been discussed from various points of view, with no conclusion having been drawn as yet. There is an opinion to the effect that, in the cas where the chemical structure is clear and when a product of high purity is manufactured at all times, such should be handled in the same manner as the ordinary chemical synthetic substances and that the same should not be put within the frame of special antibiotics.

On the other hand, t ere are people who argue that those antibiotics which are used as chemicals in the chemical treatment method should not be used as food additives. There is a line of thinking that it involves such special problems as follows: They may promote the resistance of micro-organisms including pathogenic microorganisms in addition to ordinary toxicity. They may change or replace the bacterial lump in the intestines of a man. They may serve as a factor for the developmentof hyper-tension (over-sensitiveness). In addition, there is a criticism coming from the standpoint of safety against the use of antibiotic substances as agricultural chemicals.

The authors believe that there is room for the development of low-toxic substances which do not compete with medicines as food additives.

3.7. Preservatives Not Legally Designated

Since the administrative control of food hygiene has come to extend far these days, it seems that no preservatives other than those designated by law are used. However, there is no denying the fact that boric acid, urotropin, thimol and surface active agents, etc. are furtively used. In the case of boric acid, in particular, there is a great temptation to use the

same illegally in view of the fact that no acute toxicity will be created and that it is effective as a preservative for some kinds of processed fishes and marine products. Care should be exercised so as to avoid its use.

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

食品添加物の毒性とその問題点

——相　磯　和　嘉*——

1. はじめに

400 種にものぼる食品添加物が使用されている現状において，食品は果たして安全であろうかという素朴な疑問を抱いている人々は決して少なくない．中には単なる疑問だけではなく安全ではないと信じている人達もある．

[illegible]に最近[illegible]慢性毒性に関する知[illegible]深くなってきたので，加工食品の安全性評価に対する[illegible]念はいっそう高まってきたようである．

[illegible]る傾向は日本だけではなく，アメリカ，イギリ[illegible]ヨーロッパにおいても関心は一般的に深まっており，諸国家における添加物の規制はきびしくなる[illegible]ある．食品添加物についての国際的の調査協力機関は WHO，FAO ならびに UICC（国際対ガン連合）等であるが，これらの機関における安全性評価のための活動もいよいよ活発になってきている．

わが国に限定して，かかる疑念が高まる理由を考えてみるに，一方において食用色素等で代表される添加物の使い過ぎが目だっていること，他方において食品工業の当事者のしっ[illegible]法精神の欠除に対する不信がある．その[illegible]には添加物の毒性に対する一般的知識の不足[illegible]一方の被害者意識[illegible]が入り交っていることもみ[illegible]ことができない．

[illegible]体[illegible]ないが，我は自[illegible]っ[illegible]の疑念が起[illegible]ないのが残[illegible]であ[illegible]して恐れられているほど有害なものではなく，科学的に平静心をもって評価するならば，現状ではほぼ安全性は保持されているとみて差し支えないと思う．

400 種に及ぶ添加物の大部分はその化学的性質からみて健康に有害に働く懸念のない物質である．ただ若干の添加物が現在の段階である種の懸念があるかもしれないという状況にある．この容疑は人工着色料，防腐剤，漂白剤，人工甘味料等に向けられている．ここでは食用色素と防腐剤について，現時点における毒性に立脚してみたとき，その毒性の問題点はどのようなものであるかを述べてみよう．ただし化学[illegible]には専門的の専門用語が多いので，そ[illegible]は食品添加物の指定および使用基準の設定の際の[illegible]調査会の[illegible]基準（食品衛生研究 15,10 号，3，1965）を参照されたい．

2. 食用色素

2·1 食用色素の現状

今日のような慢性毒性試験を経ないで，合成染料のなかから食用色素をえらんだ時代には，当然毒性の高い物質が混入していた．ピクリン酸，ジニトロクレゾール，メチルバイオレッド，オーラミン，ローダミン等が急性中毒を起こす危険のある色素として，[illegible]加工行政の変遷の経過のなかで使用が禁止されていった．また人工着色料はその製造の過程で[illegible]の他[illegible]混入するおそれがあるので[illegible]に関する告示」を公布して製品ごとに[illegible]検査して合格証紙を貼布しなければならないこととした．

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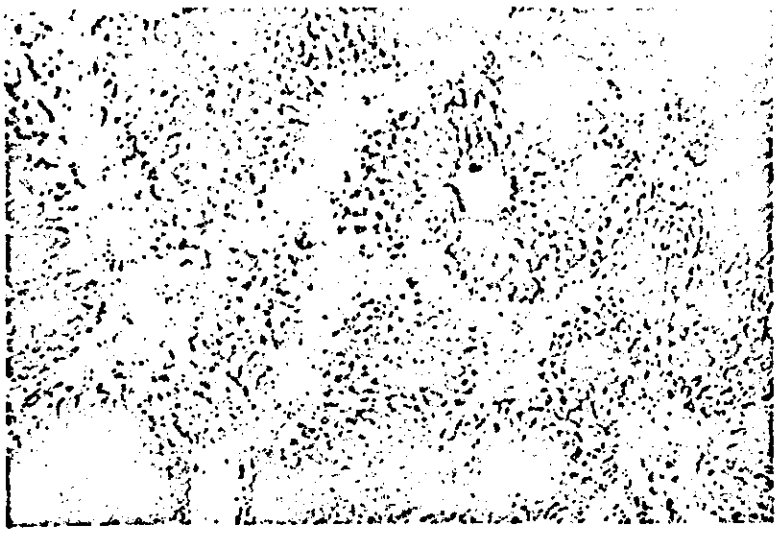
Toxicity and safety of food additives.



第2
オイルレッド（黄色3号）を0.1%に添加した[illegible]ットを12週飼育したときの肝臓組織，小葉の中に[illegible]した部分が偽小葉を作って，る

ため添加飼料と無添加飼料を断続的に行なった．0.1～0.25%添加飼料を一定期間投与したのち無添加飼料にかえ，これを繰り返して1年6カ月に及んだ．その全部の結果はまだ集計してないが，これまでの結果では時日の経過とともに病変は進行するが肝硬変に移行するものは少ない．しかしところどころに肝細胞の異型増殖，腺腫様増殖がみられるようになり，発癌の兆候がみられる．文献によれば本色素の0.02%以下の投与では毒性は顕著に現われず，肝の腫瘍発生も認められないとしているが，筆者らの方法により比較的高濃度のものを断続的に投与することでその発癌性を立証できるのではないかと考えている．

とまれ0.1%の添加量で毒性を示すことはこの色素が蓄積性があって到底安全性を確保できるものではないといいうる．この種の毒性を示すものに Oil Yellow AB（黄色2号），Oil Yellow OB（黄色3号）等があり，この2つはナフチルアミンのアゾ化合物であって古くから発癌性が疑われていたものである．これら3つの脂溶性色素が本年1月から指定を取り消されたのはかしろ当然の措置といわなければならない．

3. 防　腐　剤

防腐剤はそれぞれ作用機作は異なるにしても腐敗微生物の増殖を抑制する働きを有する点で共通に細胞毒性（cytotoxicity）をもつ．したがって概念的には毒性のある化学物質であるべきはずであり，その限りにおいて安全性の点から他の添加物よりいっそうきびしく論議されなければならない．ただすでに理解されているように，腐敗微生物には確実に阻害効果を示しながら，それを摂取する生体の細胞，組織には究局においてきわめて温和な作用しか示さないような物質が存在するので，防腐剤としてはこのような薬剤が選択されているのである．

微生物には harmful，生体細胞には harmless であるためにはいくつかの条件が必要であるが ① 生体内で完全に代謝されて無害な最終産物となって尿から排泄される．② 蓄積作用がなく，摂取された物質はより低毒性物質に変化しながら全部が尿から排泄される．③ 毒性のある物質でも5 ppm 以下の添加量で有効であり，食品中にながく残留せずまたは腸管から吸収されることなく糞便とともに排泄される，等の性状の1つを保有するならば，許容量の概念を導入することによって，安全性の確保される防腐剤の存在をわれわれは容認しうるのである．

それにしても Antimicrobials については国際的な毒性評価の基準はきびしく，WHO, FAO 専門委員会の検討に耐えてきた物質はまことに数少ない．

わが国においては食糧資源，食生活の慣習，気候条件，国民所得の低位等の理由から多くの食品が防腐剤の使用に頼らねばならない事情もあって，今日防腐剤はやや使われすぎているきらいがないでもない．以下わが国の法定防腐剤についてその毒性の程度を記載してみよう．

3·1 サリチル酸

Salicylic acid および salicylate は経口的に摂取されると腸管から容易に吸収され全身を経て尿から排泄される．ラットの一回経口投与による急性毒性 LD_{50} は1,500～2,000 mg/kg で毒性は高くないようにみえるが，蓄積作用があるために 500 mg/kg を4～21日間[illegible]すると半数死亡するし，600 mg/kg では10日以内に全例が死亡する．幼弱ラットだと0.5%に飼料に添加したとき6週間以内に成長阻害がみられる．[illegible]害作用として，ラットに1日，100 mg の本剤を[illegible]

ると 20 日で強い低トロンビン血症を呈する．

人の場合にはサリチル酸とその製剤は医学品として古くから用いられているものでその副作用はよく知られており，本品の過剰投与による中毒を Salicylism と呼んでおり中枢神経毒としての症状がみられるほか，酸－アルカリ平衡の破たん，代謝障害，肝，腎の器質的変性等もみられる．人の致死量はおそらく 20–30 g であろうとされている．

しかし長期に投与された場合の長期慢性毒性実験はふしぎと記載がない．そのため防腐剤としての決定的な毒性評価はできないのであるが WHO, FAO の専門委員会の報告では添加物としては適当でないとしている．

わが国では日本酒に 0.25 g/l に添加が許可されているが，毎日 1 l の日本酒を飲む人は 250 mg のサリチル酸を摂取するので人の致死量の 100 分の 1 に当たるが，ラットの致死量 600 mg/kg を人に換算すると 30 g となり 100 倍の安全係数をとると 300 mg となるのでぎりぎりの線となり問題となりそうである．特にアルコールとともに飲用した場合のサリチル酸の毒性は十分検討してみなければならないし，この問題が今日までなおざりにされていたのは理解できないところである．

3·2 安息香酸，安息香酸ソーダ，パラオキシ安息香酸エステル類

Benzoic acid は生体内に速やかに吸収されるが代謝されて無害な馬尿酸あるいは結合グルクロン酸として尿中から完全に排泄される．この吸収，排泄機構はラットで C^{14} でラベルした安息香酸を用いた実験で証明されている．

この生化学研究によっても本品の低毒性を予想できるが Sodium benzoate の LD_{50} は 2,700～2,000 mg/kg（ラット，マウス，犬）である．慢性毒性実験では 0.5% と 1.0% の本品添加飼料でラットの一生涯（約 2 年半）を飼育した報告があるが 1% 添加でも成長阻害はなく死亡率も対照群と差異がなく，解剖所見からも毒性がみられなかった．

サリチル酸と安息香酸がオルトに OH がついただけで生体代謝に相違ができて毒性に強弱があるのは興味ある事実である．

パラオキシ安息香酸エステル類についてはメチル，エチル，プロピルエステルについて詳細な毒性実験があり，LD_{50} はマウスで 2,000～3,700 mg/kg の範囲にある．慢性毒性実験では 2～8% 添加飼料でラットを 96 週飼育した結果，8% の場合プロピルエステルでは体重の増加は阻害をうけたが 3 つのエステルについて 2% 濃度では no effect level であり，著しく低毒性であることが知られている．このように安息香酸，安息香酸ソーダ，パラオキシ安息香酸エステル類は防腐剤としては比較的安全性が高いものと考えて差し支えない．

3·3 ソルビン酸とプロピオン酸塩類

Sorbic acid は生体内でカプロン酸と同じ型式で β-oxidation によって代謝され最終的に CO_2 と H_2O になる．動物はこの際カロリー源としてソルビン酸を利用できる．またラットの実験で必須脂肪酸の代謝拮抗物として使われることのないことも明らかにされている．このような生化学的所見からみて本品も低毒性物質と推定されるわけである．

本品の LD_{50} はラットで 10,500 mg/kg という実際上無毒ともいえる値を示している．慢性実験では 5% の添加飼料で 1,000 日の feeding で成長阻害はなく，他の中毒性変化がみられなかったので no effect level は 5% (2,500 mg/kg) となる．

プロピオン酸塩類は生体内で正常脂肪酸と同じ経路で代謝され利用される．マウスの実験で C^{14} でラベルしたプロピレン酸は体脂肪の先駆物質として見い出され，さらに C^{14} は CO_2, glucose, succinate, protein 等に分布してゆくこと等がたしかめられている．

プロピオン酸の急性毒性は LD_{50} がラットで 2,600 mg/kg であり，15% の水溶液は点眼しても刺激性でない．

長期投与実験では 3.75% の添加飼料でラットを 1 カ年飼育しても成長阻害，死亡率の上昇はなくまた病理組織学的の検査からも中毒所見は見当たらない．これらの結果からもプロピオン酸塩類も安全性の高い防腐剤といえる．

3·4 デヒドロ酢酸

Dehydroacetic acid の急性毒性はラットで LD_{50} 値は 1,000 mg/kg，マウスで 1,270 mg/kg であり，ナトリウム塩ではラットで 570 mg/kg，マウスで 1,050 mg/kg であるからこれまで述べた物質より毒性が高い．マウスに 1.5～1.9 g/kg 投与すると死亡する．ラットの 6 カ月連続投与では 0.1 g/kg では毒性は現われないが 0.2 g/kg では成長阻害が認められ，さらに 0.3 g/kg では 2 カ月前後に死亡する．

慢性毒性試験では 0.1% 添加飼料ではラット 2 カ年の投与で毒性はみられない，猿では 0.2 g/kg で 1 年の投与で異常はなかった．no effect level は 100 mg/kg となる．この値を人に換算して 100 倍の安全係数をとると 1 日に 50 mg の DHA が最大許容量となるので，現在許可されている食品とその使用基準の範囲内では安全性が確保されているが，あまり余力はないので指定外の食品への不法使用は許されない．これまで述べてきた保存料の LD_{50} 値と no effect level を一覧表として掲げた．

Table 1 第1表　指定保存料の毒性比較

a 保存料	b 急性毒性，DL_{50} mg/kg	c 慢性毒性試験によるラット安全量
Salicylic acid	1,500—2,000 (rat)	1 信頼すべき慢性毒性実験の資料がない
Benzoic acid	2,700 (rat)	2 飼料中に 1% (500 mg/kg)
Sodium benzoate		
Methyl p-hydroxybenzoate	2,000 (mouse)	3 飼料中に 2% (1,000 mg/kg)
Propyl p-hydroxybenzoate	3,700 (mouse)	4 飼料中に 2% (1,000 mg/kg)
Propionate of sodium & calcium	2,600 (rat) (propionic acid)	5 飼料中に 3.75% (2,000 mg/kg)
Sorbic acid	10,500 (rat)	6 飼料中に 5.0% (2,500 mg/kg)
Dehydroacetic acid	1,000 (rat) 1,270 (mouse)	7 飼料中に 0.1% (50 mg/kg)

3·5 フリルフラマイド (AF-2)

ニトロフラン誘導体のある種のものを食品防腐剤（殺菌剤）として使用しているのはわが国だけであるので，[illegible]細の資料は国内の研究に頼るほかない．昨年[illegible]マイドが新たに指定となり，従来のニトロフ[illegible]Z[illegible]は指定取り消しとなったが，毒性[illegible]ニトロフランよりも低いことが判明した結[illegible]．

抗菌性ニトロフランは既述の保存剤とは趣きを異にして，本格的の抗菌性物質であってその抗菌力はきわめて高い．フリルフラマイドの場合は多くの腐敗細菌に5 ppm 以内で発育阻止効果を発揮する．したがって添加濃度を 5 ppm 以下に止めることが可能である．

ところが毒性は低い物質とはいえず，急性毒性で LD_{50} [illegible]は 570 mg/kg 程度である．食餌に 0.1% に添加[illegible]も3カ月以内には成長阻害はなく，0.2% の添加[illegible]に耐える．0.01～0.1% 濃度でラットを1～2年飼育しても主要臓器に顕著な中毒性変化を招[illegible]ることはない．0.2% 以上の投与で，ニトロフラゾ[illegible]の場合にみられるごとき睾丸の萎縮，精子形成障害は発現しないが，肝の軽度の肥大がみられる．ただしこの肝肥大は肝実質細胞の平衡のとれた増大であって，異型増殖とは異なるもので長期飼育ののちでも腫型化する傾向は認められていない．阪大の宮地徹教授は本品の no effect level を 0.01% の添加濃度としているが，筆者は[illegible]投与成績ではあるが 0.02% は安全量と考えてよいと思っている．

0.01% をラットの安全量とすると 5 mg/kg となり人に換算して 100 倍の安全係数をとると1日量は 2.5 mg となる．食品への添加濃度を 20 万分の1とすると，許される添加食品重量は 500 g になるので現在の許可食品の範囲内では安全性は確保されている．しかしこの化学物質が[illegible]内でどのように代謝され，代謝産物がどのような[illegible]を持つかという研究が今後望ましいことである．またこのような本格的な抗菌物質は数少ない特定の食品に微量に添加するように限定しておくことが必要なことかもしれない．

3·6 抗生物質

抗生物質を鮮度保持の目的で食品に添加することの可否については種々の観点から論ぜられており，いまだ結論に達していない．化学構造が判明しており，純度の高い製品が常に製造できるものについては，一般化学合成物質と同様に取り扱い，特別に抗生物質という枠にはめる必要はないという見解も成立する．これに対し化学療法剤に使用する抗生物質は食品添加物として使用すべきではないとする反論もある．一般毒性のほかに病原微生物を含めて微生物の耐性化を促進するかもしれないこと，人の腸内細菌叢を変更もしくは交代させるかも知れないこと，過敏症の発生要因となるかもしれないこと等の特殊の問題があるとする考え方も根強く存在している．さらに農業として抗生物質が使われている現状に対する安全性の立場からの批判もある．

著者は医薬品と競合しない低毒性抗生物質については食品添加物として開発の余地はあるものと考えている．

3·7 法定外防腐剤

食品衛生監視行政が行き届くようになったので，今日では法定防腐剤以外の薬剤を使用することはほとんどなくなったと思われるが，なお，ホウ酸，ウロトロピン，チモール，界面活性剤等がひそかに使用される事例が絶無ではない．特にホウ酸については急性毒性が起こりにくいこと，ある種の魚介類加工品の防腐に有効なこと等の理由で不法利用の誘惑にかられるかもしれず，大いに警戒を要するところである．

× × ×

36

Solution to the separator,
e column, discarding the
ide Solution to the column,
an, and receive the eluate
mn for the next run, pass
ough the column, and then
rashings.) To the beaker
15 to 20 minutes. Add
on with stronger ammonia
tals, stir to dissolve, and
.S., with stirring, and stir
ith occasional stirring for
the beaker with suction
orted in a 25 mm. porce-
ed with a suspension of
beaker have been trans-
10-ml. portions of a 1 in
e, passing the washings
ive 5-ml. portions of the
precipitate to the beaker,
the beaker, and dilute
de from a buret until the
o 8 ml. in excess. Add
alkali with 0.1 *N* nitric
hydroxide to the first
ence between the total
nd the volume of nitric
l., of 0.1 *N* sodium hy-
ate complex. Calculate
le taken by the formula

r 1 hour.

water meets the require-

nd proceed as directed

5 ml. of water meets the
20, using 20 mcg. of lead

in 100 ml. of hot water,
ash the insoluble residue
nd weigh.

water meets the require-
5 mcg. of lead ion (Pb) in

130a

Packaging and storage. Store in tight containers.
Functional use in foods. Texturizer.

SORBIC ACID

2,4-Hexadienoic Acid

$CH_3CH{=}CHCH{=}CHCOOH$

$C_6H_8O_2$ Mol. wt. 112.13

DESCRIPTION

A white, free-flowing powder with a characteristic odor. It is slightly soluble in water. One gram dissolves in about 10 ml. of alcohol and in about 20 ml. of ether.

IDENTIFICATION

A. To 2 ml. of a 1 in 10 solution of the sample in alcohol add a few drops of bromine T.S. The color is discharged.

B. A 1 in 400,000 solution in isopropanol exhibits an absorbance maximum at 254 ± 2 mμ.

SPECIFICATIONS

Assay. Not less than 99.0 percent and not more than the equivalent of 101.0 percent of $C_6H_8O_2$, calculated on the anhydrous basis.

Melting range. Between 132° and 135°.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).
Heavy metals (as Pb). Not more than 10 parts per million (0.001 percent).
Residue on ignition. Not more than 0.2 percent.
Water. Not more than 0.5 percent.

TESTS

Assay. Dissolve about 250 mg., accurately weighed, in 50 ml. of anhydrous methanol which previously has been neutralized with 0.1 *N* sodium hydroxide, add phenolphthalein T.S., and titrate with 0.1 *N* sodium hydroxide to the first pink color which persists for at least 30 seconds. Each ml. of 0.1 *N* sodium hydroxide is equivalent to 11.21 mg. of $C_6H_8O_2$.

Melting range, page 931. Determine as directed for *Class Ia*, but heat at a rate of rise of 1° per minute until the melting is complete.

Arsenic. A *Sample Solution* prepared as directed for organic compounds meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. Prepare and test a 2-gram sample as directed in

Method II under the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Residue on ignition, page 945. Ignite 2 grams as directed in the general method.

Water. Determine by the *Karl Fischer Titrimetric Method*, page 977.

Packaging and storage. Store in tight containers protected from light, preferably at a temperature not exceeding 38°.

Functional use in foods. Preservative.

SORBITAN MONOSTEARATE

DESCRIPTION

A mixture of partial stearic and palmitic acid esters of sorbitol and its mono- and dianhydrides. It is manufactured by reacting edible commercial stearic acid (usually containing associated fatty acids, chiefly palmitic) with sorbitol. It is a light cream to tan colored, hard, waxy solid with a bland odor and taste. It is soluble at temperatures above its melting point in toluene, dioxane, carbon tetrachloride, ether, ethanol, methanol, and aniline. It is insoluble in cold water, and in mineral spirits and acetone, but is dispersible in warm water and soluble, with haze, above 50° in mineral oil and in ethyl acetate. It conforms to the regulations of the federal Food and Drug Administration pertaining to specifications for fats or fatty acids derived from edible sources.

IDENTIFICATION

A. The fatty acid residue obtained in the *Assay* has an *Acid Value* between 200 and 212 (*Method I*, page 902) and a *Solidification Point* not below 53°.

B. *Apparatus.* Assemble a suitable apparatus for ascending thin-layer chromatography. Prepare a slurry of chromatographic silica gel and water (1 gram to 2 ml.), apply a uniformly thin layer to glass plates of convenient size, dry in the air for 10 minutes, and activate by drying at 100° for 1 hour. Store the cooled plates in a desiccator over anhydrous calcium sulfate until ready for use.

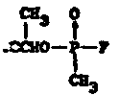
Sample Solution. Transfer 500 mg. of the polyols obtained in the *Assay* into a 2-ml. volumetric flask, dissolve, dilute to volume with water, and mix.

Standard Solution. Transfer 50 mg. each of sorbitol, 1,4-sorbitan, and isosorbide into a 1-ml. volumetric flask, dissolve, and dilute to volume with water, and mix.

Procedure. By means of the template provided with the apparatus, mark the sample spot area 1.5 cm. from the bottom of the plate, then

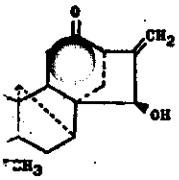
294a

onofluoridic acid 1,2,2-trimethyl-
ethylphosphoryl fluoride; pina-
oridate. $C_7H_{16}FO_2P$; mol wt
.85%, F 10.43%, O 17.56%, P
edt, *Acta Physiol. Scand.* 25,
otar 16, 131 (1950). G. Schrader,
ztizide auf Grundlage organischer
ndungen [Monographie Nr. 62
und Chemie-Ingenieur-Technik
. C. Saunders, *Some Aspects of*
Action of Organic Compounds
Fluorine (Cambridge, 1957),



2 mg/100 g; dermally in mice:
iafsky, *Toxicol. Appl. Pharmacol.*
for man may be as low as 0.01
: 31, 4676 (1953).
: cholinesterase inhibitor. Toxic
re intense than, parathion, *q.v.*

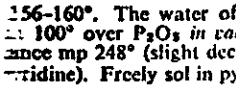
: zongorine. $C_{22}H_{31}NO_3$; mol
8.74%, N 3.92%, O 13.43%.
Popov, *Ranunculaceae:* Yunu-
R.) 18, 515 (1948); Kuzovkov,
6 (1955). Identity with napello-
nchel Khim. 28, 2283 (1958); 29,
gasawa, *Chem. Pharm. Bull.* 9,
configuration: Okamato *et al.*,



$=_D^{20}$ −135.4°. Absorption max:

mp 257–258°. $[\alpha]_D^{20}$ −114°

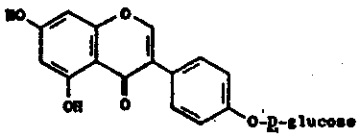
4'-glucosidorhamnoside. C_{27}-
56. H_2O 8.54%. Anhydr form
56.05%, H 5.23%, O 38.72%.
ica L., *Leguminosae*. Isoln and
. *Ber.* 75B, 482 (1942). The
inose.



O-D-glucose-L-rhamnose

156–160°. The water of crystn
100° over P_2O_5 *in vacuo* for
nce mp 248° (slight decompn).
idine). Freely sol in pyridine;
; slightly sol in boiling water.
rple color with ferric chloride.

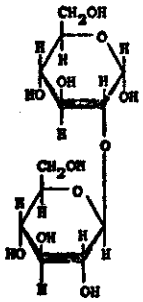
ydroxyisoflavone-4'-D-glucoside;
$H_{20}O_{10}$; mol wt 432.37.
%. From the green pods of
osae: Charaux, Rabaté, *Bull.*
8). Structure: Zemplén *et al.*,
A. 46, 8104h (1952). Synthe-
im. Acad. Sci. Hung. 4, 383
(*L*on) 1954, 518. Hydro-
is.
298°. $[\alpha]_D^{20}$ −47° (in pyridine).
ine). Absorption max in abs



O-D-glucose

ethanol: 262 mμ. Sparingly sol in water, alcohol, acetic acid; more sol in hot alcohol, hot acetic acid; sol in pyridine, dil alkalies; practically insol in ethyl acetate, acetone. Ferric chloride gives the color of red wine to alcoholic solns which turn orange after addition of a few drops of Na-carbonate soln.

Sophorose. 2-*O*-β-D-Glucopyranosyl-α-D-glucose. $C_{12}H_{22}O_{11}$; mol wt 342.30. C 42.10%, H 6.48%, O 51.42%. From pods of *Sophora japonica* L., *Leguminosae:* Rabaté, *Bull. Soc. Chim. France* 7, 565 (1940); Clancy, *J. Chem. Soc.* 1960, 4213; M. J. Clancy in R. L. Whistler, M. L. Wolfrom, *Methods in Carbohydrate Chemistry* Vol. 1 (Academic Press, New York, 1962), pp 345–349. Structure and synthesis: Coxon, Fletcher, *J. Org. Chem.* 26, 2892 (1961).



Monohydrate, $C_{12}H_{22}O_{11}.H_2O$, needles from 80% aq methanol, mp 196–198°. $[\alpha]_D^{18}$ +19° (c = 1.2 in water).

Octa-*O*-acetyl-β-sophorose, $C_{28}H_{38}O_{19}$, needles from ethanol, mp 193–194°. $[\alpha]_D^{18}$ −3.2° (c = 2.5 in chloroform).

Sorbic Acid. *2,4-Hexadienoic acid;* 2-propenylacrylic acid. $CH_3CH{=}CHCH{=}CHCOOH$; mol wt 112.12. $C_6H_8O_2$; C 64.27%, H 7.19%, O 28.54%. May be obtained from berries of the mountain ash, *Sorbus aucuparia* L., *Rosaceae* where it occurs as the lactone, called parasorbic acid: Hofmann, *Ann.* 110, 129 (1859). Synthesis by condensing crotonaldehyde and malonic acid in pyridine soln: Doebner, *Ber.* 33, 2140 (1900); Allen, Van Allan, *Org. Syn.* coll. vol. 3, 783 (1955); by condensing crotonaldehyde and ketene in the presence of boron trifluoride; Hagemeyer, Jr., *Ind. Eng. Chem.* 41, 768 (1949). Prepn from 1,1,3,5-tetraalkoxyhexane: Parker, MacLean, U.S. pat. 2,921,090 (1960 to Celanese). Several other syntheses: Fernholz, Mundlos, Ger. pat. 1,049,852 (1959 to Farbwerke Hoechst); U.S. pat. 3,021,365 (1962 to Farbwerke Hoechst). Purification: Fernholz, Ger. pat. 1,044,803 (1958 to Farbwerke Hoechst). The *trans,trans*-isomer is usually obtained and is the commercial product.

Needles from water, mp 134.5°. Should be stored at temps below 40°. bp 228° (decompn). Vapor pressure at 20° = <0.01 mm, at 143° = 50 mm. Flash pt 260°F (127°C). K at 25° = 1.73 × 10^{-5}. Solubility in water at 30° = 0.25%, at 100° = 3.8%, in propylene glycol at 20° = 5.5%, in abs ethanol or methanol = 12.90%, in 20% ethanol = 0.29%, in glacial acetic acid 11.5%, in acetone 9.2%, in benzene 2.3%, in carbon tetrachloride 1.3%, in cyclohexane 0.28%, in dioxane 11.0%, in glycerol 0.31%, in isopropanol 8.4%, in isopropyl ether 2.7%, in methyl acetate 6.1%, in toluene 1.9%.

Also used as the sodium salt. Prepn: Horn, Fernholz, Ger. pat. 1,045,390 (1958 to Farbwerke Hoechst).

USE: Mold and yeast inhibitor. Fungistatic agent for foods, especially cheeses. To improve the characteristics of drying oils. In alkyd type coatings to improve gloss. To improve milling characteristics of cold rubber. LD_{50} orally in rats: 7.36 g/kg. The use of calcium sorbate is described in U.S. pat. 3,139,378 (Gooding, 1964 to Corn Products Co.). *See also* Potassium Sorbate. *Human Toxicity:* Practically nontoxic. May cause slight skin irritation.

Sorbic Alcohol. *2,4-Hexadien-1-ol;* 1-hydroxy-2,4-hexadiene; Hexene-Ol; Hexakose; hexadenol. $CH_3CH{=}CHCH{=}CHCH_2OH$; mol wt 98.14. $C_6H_{10}O$; C 73.43%, H 10.27%, O 16.30%. Prepd from sorbic aldehyde by means of aluminum isopropylate in isopropanol: Reichstein, Amman, Trivelli, *Helv. Chim. Acta* 15, 264 (1932).

Long needles. Agreeable odor of new mown grass. mp 30.5–31.5°. bp_{12} about 80°. Volatile with steam. Insoluble in water. Soluble in alcohol, ether, oils. Not stable to air; must be sealed in evacuated ampuls for storage.

Diphenylurethan, $C_{19}H_{19}NO_2$, crystals from petr ether, mp 78–79°.

3,5-Dinitrobenzoate: Yellowish needles from petr ether, mp 85°.

Sorbitol. D-Sorbitol; D-glucitol; L-gulitol; sorbit; Sorbol; Sorbicolan; Sorbo; Sorbostyl; Nivitin; Cholaxine; Karion; Sionit; Sionon; Sorbilande; Diakarmon. $C_6H_{14}O_6$; mol wt 182.17. C 39.56%, H 7.75%, O 52.70%. First found in the ripe berries of the mountain ash *Pyrus aucuparia* Ehrh. (L.) (*Sorbus aucuparia* L.), *Rosaceae*. Occurs also in many other berries (except grapes) and in cherries, plums, pears, apples, seaweed and algae. Has been detected in blackstrap molasses. Isoln from berries: Embden, Griesbach, *Z. Physiol. Chem.* 91, 268 (1914). Prepd industrially from glucose by high pressure hydrogenation or by electrolytic reduction: Boye, *Chemiker-Ztg.* 82, 657–660 (1958); Fedor *et al.*, *Ind. Eng. Chem.* 52, 282–286 (1960).

Needles with ½ or 1H_2O. Sweet taste: About 60% as sweet as sugar (w/w). In the healthy human organism one gram of sorbitol yields 3.994 calories which is comparable to 3.940 calories from 1.0-g of cane sugar. Seventy percent of orally ingested sorbitol is converted to CO_2 without appearing as glucose in the blood: Adcock, Gray, *Biochem. J.* 65, 554 (1957). The hydrated crystals melt somewhat below 100°. When completely anhydr mp 110–112°. $[\alpha]_D^{20}$ −2.0° in H_2O. In the presence of molybdate the rotation is reversed and increased to +56°. Freely sol in water (up to 83%). High percentage sorbitol solns are much more viscous than corresponding glycerol solns. Quite sol in hot alcohol, sparingly sol in cold alcohol. Also sol in methanol, isopropanol, butanol, cyclohexanol, phenol, acetone, acetic acid, dimethylformamide, pyridine, acetamide solns. Practically insol in most other organic solvents. Not attacked in the cold when mixed with dil acids, alkalies or mild oxidizing substances. Ka at 17.5° = 2.5 × 10^{-14}. pH about 7.0. R_f value (water–butanol) = 0.124. A commercial 70% aq soln may have the following characteristics: d_{20}^{20} 1.2879; n_D^{25} 1.45831; $[\alpha]_D^{20}$ −2.10°; bp_{760} 105°; pH between 6 and 7. Viscosity at 25° = 110 centipoises (cp). The following table compares glycerol and sorbitol solns:

%	d_4^{20} sorbitol	d_4^{20} glycerol	cp at 20° sorbitol	cp at 20° glycerol
5	1.019	1.011	1.230	1.143
10	1.038	1.023	1.429	1.311
15	1.062	1.035	1.777	1.517
20	1.077	1.047	2.061	1.769
25	1.099	1.060	2.689	2.095
30	1.124	1.073	3.655	2.501
40	1.153	1.099	5.323	3.750
50	1.198	1.127	11.09	6.050
60	1.249	1.154	35.73	10.96
70	1.299	1.182	185	22.94
83	1.391	1.218	>10,000	111

USE: In manuf of sorbose, ascorbic acid, propylene glycol, synthetic plasticizers and resins; as humectant (moisture conditioner) on printing rolls, in leather, tobacco. In writing inks to insure a smooth flow and to prevent crusting on the point of the pen. In antifreeze mixtures with glycerol or glycols. In candy manuf. In pharmaceutical

ORAL, SUBCUTANEOUS AND INTRATRACHEAL ADMINISTRATION OF CARCINOGENIC LACTONES AND RELATED SUBSTANCES: THE INTRATRACHEAL ADMINISTRATION OF CIGARETTE TAR IN THE RAT

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It has now been possible to test 33 compounds with a lactonic or related structure, of which 25 have proved on subcutaneous injection to be capable of inducing the formation of tumours of the connective tissue elements of rats (Dickens and Jones, 1961; 1963*a*, *b*; 1965). The carcinogenic substances are for the most part characterised by chemical structures which indicate their reactive nature. This may be due to:

a. Strain in the lactone ring, particularly in the 4-membered rings.
b. Conjugation of double bonds, within and outside the ring, with the lactonic carbonyl group.
c. Conjugation of double bonds even in linear compounds.
d. Presence of the anhydride ring of dicarboxylic acids.

Where these features were absent, or where the substances caused death of the animals before tumours could be expected to appear, it was not possible to show a carcinogenic action in members of this series.

Dickens and Cooke (1965) studied the rates of hydrolysis and of interaction with cysteine of these substances and showed that, with the exception of aflatoxin and N-ethyl maleimide, the rates of reaction with cysteine could be correlated in a very approximate manner with their carcinogenic activity.

It is becoming clear that carcinogens belonging to this chemical class might be a pertinent factor in human cancer. Many compounds of this general type are widespread in animal and vegetable tissues, are produced by some saprophytic fungi infecting foodstuffs, and may possibly be produced during the burning of tobacco (Dickens, 1963). Some of them are useful antibiotics, and it is unfortunate that the very molecular structure which makes them active agents against bacteria and fungi is probably also that which makes them active as carcinogens. In relation to this problem it is important to know whether such compounds are carcinogenic not only when injected but when taken into the stomach and lungs, these being the routes by which some substances related to those dealt with in these studies are liable to be ingested by man.

This paper reports the results of testing four further substances chosen because of the similarity of their structure to other compounds which have already proved to be carcinogenic. They are sorbic acid and dehydroacetic acid—salts of which

are widely used as fungistatic agents—sterigmatocystin, a mould metabolite, and geduin, isolated from African hardwoods. Sorbic acid and dehydroacetic acid have also been administered by mouth, as well as some other substances which have previously been shown to be carcinogenic by injection. We also report the result of an experiment with a series of known carcinogens designed to investigate their effects on the lungs of rats. For comparison, cigarette smoke condensate has been similarly administered intratracheally to groups of rats over a long period.

EXPERIMENTAL

Materials

The sources and characteristics of aflatoxins B_1 and G_1, (+)-parasorbic acid, β-propiolactone, penicillic acid and methylprotoanemonin have already been described by Dickens and Jones (1961; 1963*a*, *b*; 1965).

* *Sorbic acid* (trans-trans-2,4-hexadienoic acid), *Dehydroacetic acid* (II) and *benzopyrene* were purchased from L. Light & Co., Colnbrook, Bucks.

Sterigmatocystin (III) was kindly provided by Professor J. C. Roberts, Department of Chemistry, University of Nottingham, through Dr. A. J. Fenell of the Tropical Products Institute, London (Davies, Kirkaldy and Roberts, 1960; Bullock, Roberts and Underwood, 1962; Roberts and Underwood, 1962).

Geduin (IV) was a gift from Professor C. W. L. Bevan, Department of Chemistry, University of Ibadan, Nigeria (Bevan *et al.*, 1962).

Cigarette smoke condensate was made available by Dr. H. R. Bentley through the kind cooperation of the Tobacco Research Council, London (Director: Mr. G. F. Todd). The fraction used was the neutral material resulting from the standard T.R.C. fractionation process.

Animal experiments

Twice-weekly subcutaneous injections of substances were made into the right flanks of groups of 6 rats weighing initially 100 g. for 65 weeks, or for as long as the supply lasted in the case of sterigmatocystin. The substances were injected as solutions in arachis oil, or as fine suspensions for sterigmatocystin and geduin. A control group of 6 rats was injected with the oil alone. Sorbic acid, dehydroacetic acid and geduin were injected in repeated doses of 2 mg. in 0·5 ml. oil and sterigmatocystin in doses of 0·5 mg. in 0·5 ml. oil. The mixed aflatoxins were injected at a dose of 2 μg. in 0·5 ml. oil and preliminary results relating to this group have already been reported by Dickens and Jones (1965).

Oral administration was achieved by supplying solutions of the substances to groups of 6 rats daily to drink for a period of 64 weeks. Concentrated stock solutions in water were stored at 4° and diluted with tap water to replenish the water bottles. Mixed aflatoxins were given to two groups of rats at concentrations of 100 μg. and 100·0 μg. 100 ml. water, parasorbic acid to two groups at 1 mg. and 200 μg. 100 ml. and sorbic acid and dehydroacetic acid to one group of rats each at 10 mg. 100 ml. The latter two substances were neutralised with sodium bicarbonate to pH approximately 5 in order to bring them into solution. By laparotomy under ether anaesthesia the animals were examined at intervals for evidence of liver tumours. A complete search for tumours of all organs was made on animals when they died and on all those surviving at 100 weeks.

* For structural formulae I, II, III and IV see p. 140.

Solution of the sparingly soluble aflatoxins for use in the drinking water was achieved by adding the solution of 25 mg. mixed aflatoxins ($B_1 + G_1$) in 20 ml. warm absolute ethanol to a large volume of water (1980 ml.). After allowing to stand at 4° C. for several weeks, the amount remaining in solution was determined by measurement, against a blank containing the appropriate amount of ethanol, of the ultraviolet absorption. This corresponded to 86 per cent of the total amount originally taken, i.e. 10 μg./ml. instead of 12·5 μg./ml. This stock solution was then diluted daily with water 10 or 100 times for preparation of the solutions of aflatoxin as placed in the drinking bottles.

A technique for administering a substance by intubation directly into the trachea of a rat has been used to investigate the induction of lung cancer in treated animals. Modification of a technique (Littlewood and Platts, 1953) kindly described to us by the Staff of Messrs. Fison's Toxicological Laboratory, Saffron Walden, allowed administrations to be made at the rate of one rat per minute. The apparatus consisted of a sheet of perspex attached upright to a heavy wooden base and having its upper half inclined towards the operator. Rats, treated with atropine sulphate to prevent salivation were anaesthetised with ether and suspended by their fore limbs by loops of string. Suspension could be rapidly effected by fastening the loops round the limbs by a sliding piece of polythene tubing, provided that the two free ends of the loops passed through a hole in the perspex small enough to grip the double strand of string without tying. The head of the rat was pressed against the perspex by a rubber band which passed round it and under the upper incisors. For intubation, the animal's tongue was pulled forward and the inside of the mouth illuminated by a laryngoscope, of the type used for children, with the speculum removed. A curved, blunted serum needle, attached to a tuberculin syringe in an Agla micrometer, was passed between the vocal chords for approximately one inch into the trachea and the required amount of substance delivered. Up to 40 μl. of material was placed in this way at the lower end of the trachea and from this site it was expected to pass subsequently into the lungs. By fluorescent microscopy it has been possible to show that benzopyrene placed in the trachea passed into the lungs within a few hours while little or none appeared to be regurgitated into the mouth. Arachis oil alone was administered to 6 rats as a control by the same technique and both experimental and control rats were given tetracycline in the drinking water (100 mg./l.) throughout the course of the experiment to minimise the risk of intercurrent infections causing their death. Intratracheal administrations were made twice-weekly for 30 weeks with very good survival and the rats were examined when they died or at 100 weeks after the first administration, particularly for lesions of the respiratory and intestinal tracts. β-Propiolactone, penicillic acid, aflatoxins B and G, and benzopyrene were given intratracheally in doses of 0·3 mg., and methylprotoanemonin in doses of 0·6 mg. dissolved or suspended in 30 μl. of arachis oil, each to 6 male rats.

The undiluted neutral fraction of tobacco smoke condensate (30 μl. doses) was instilled intratracheally once weekly and 3 times weekly to separate groups, each of 10 female rats, throughout the course of one year. As controls of these groups, 6 female rats were treated with atropine, ether and tetracycline but were not given any intratracheal treatment.

All tissues that appeared to be abnormal by macroscopic observation were examined histologically as described by Dickens and Jones (1961).

RESULTS

In the control injection experiment four rats survived for longer than 72 weeks, three eventually dying without a tumour, but one rat which died at 81 weeks carried a large sarcoma-like tumour, not histologically malignant, at the site of the injections (Table I). Two groups, each of 20 mice (not included in the Table) were also injected with the same arachis oil twice-weekly for 65 weeks but only 16 of these survived for longer than 69 weeks and in the survivors one malignant fibrosarcoma and one metastasising mammary adenoma were found at the site of injections. (Rats also withstood the repeated intratracheal administration of the oil extremely well without developing tumours in the lungs or elsewhere; see Table III.)

Subcutaneous injections of sorbic acid, sterigmatocystin and dehydroacetic acid in oil have given rise to appreciable numbers of sarcomas in the injected rats (Table I), each group including tumours which, because of the marked prolifer-

TABLE I.—*The Carcinogenic Action of Compounds Administered Twice Weekly by Subcutaneous Injection in Oil to Groups of 6 Male Rats*

Substance tested	Dose	Duration of treatment (weeks)	Earliest appearance of tumours (weeks)	Number of rats alive when first tumour seen	Number of rats with local tumours	Total period observed (weeks)	Other tumours found at autopsy
Arachis oil (controls)	(0·5 ml.)	65	81	3	1	89	None.
Aflatoxins ($B_1 + G_1$)	2 μg.	65	44	6	5	73	None.
	2 mg.	65	82	6	5	97	None.
Sorbic acid						65	Hepatoma (50 weeks) Cholangioma (62 weeks).
Sterigmatocystin	0·5 mg.	24	47	6	3		
Dehydroacetic acid	2 mg.	65	37	6	5	85	None.
Cedunin	2 mg.	65	103	1	1	103	None.

ative activity and the presence of giant multinucleate cells, are considered to be malignant. Injections of sterigmatocystin also induced a hepatocellular tumour at 50 weeks and a cholangiocarcinoma at 62 weeks in rats which also bore local sarcomas. Cedunin injections gave rise to only one sarcomatous growth after a very long time and this on histological grounds was much less malignant. One rat at 67 weeks was killed because of an infected abscess at the site of the injections. The bladder of this animal contained 18 small calculi and the bladder wall was much thickened. When examined histologically it was seen that both surfaces of the bladder wall were covered with a squamous metaplasia by invasion from the inner surface. This is considered to be a carcinoma of the bladder for this reason.

Two microgram doses of the mixed aflatoxins injected in oil twice weekly for 65 weeks induced 5 malignant sarcomas in the 6 rats which were alive at the time of appearance of the first tumour. The 6th rat which was alive when a preliminary report (Dickens and Jones, 1965) was made of this result, eventually died without developing a tumour.

Of the substances administered to rats in their drinking water (Table II), only the mixed aflatoxins have shown themselves capable of inducing tumours.

TABLE II.—*The Effect of Various Substances with Known Carcinogenic Activity Added to the Drinking Water of Rats*

Groups of 6 male rats were used and administration continued for 64 weeks in each experiment

Substance added to water	Concentration /100 ml. drinking water	Average weekly intake per rat (ml.)	Earliest appearance of liver tumours (weeks)	Number of tumours number of survivors	Other tumours found (time in weeks) and remarks
Aflatoxins (B_1 - G_1)	100 μg.	307	39	6, 6	Lachrymal gland (53)
Aflatoxins (B_1 - G_1)	10 μg.	356	66	1, 5	None
Parasorbic acid	1 mg.	300	—	—	None
Parasorbic acid	200 μg.	220			Leydig cell tumour (103)
Sorbic acid	10 mg.	312	—		None—only one rat surviving at 64 weeks
Dehydroacetic acid	10 mg.	463	—	—	None—liver necrosis in a rat alive at 103 weeks

mainly of the liver but one also of the lachrymal gland. The liver tumours were first seen in rats given the larger dose when they were first examined by laparotomy at 39 weeks. Eventually all the rats drinking water containing 100 μg. of aflatoxins/100 ml. developed hepatocellular tumours. The lachrymal gland tumour weighing 1·5 g. was also found in one of the animals of this group at 53 weeks. Only one rat given drinking water containing the lower concentration of aflatoxins, 10·0 μg./100 ml., developed a liver tumour, found at laparotomy at 66 weeks. No liver tumours were seen in rats given parasorbic acid at 1 mg./100 ml. or at 200 μg./100 ml., or given sorbic acid or dehydroacetic acid at 10 mg./100 ml. to drink. One rat which had been given parasorbic acid (200 μg./100 ml.) to drink for 64 weeks was found to have a Leydig cell tumour when killed at 103 weeks and necrosis of the livers was seen in rats which survived to 103 weeks after drinking dehydroacetic acid (10 mg./100 ml.) for 64 weeks. Intercurrent disease affected some groups in this series adversely, so that of the rats given sorbic acid to drink five died quite early in the experiment with abscesses of the lungs, pericardium and peritoneum. Only one of these rats survived for the full treatment period and was killed at 64 weeks, but no tumours were found. Three rats given 200 μg. parasorbic acid/100 ml. water to drink also died of generalised infection at the same time, though those which survived eventually died at 87, 95 and 100 weeks. None of these developed tumours of the digestive tract or related organs, but there was a testicular tumour in the last survivor.

Following the intratracheal administration of penicillic acid (0·3 mg.), methyl protoanemonin (0·6 mg.) and benzopyrene (0·3 mg.) twice weekly for 30 weeks, and tobacco tar neutral fraction (30 μl.) once weekly or three times weekly for 52 weeks, no tumours were seen in the lungs even in rats which survived to 100 weeks (Table III). All the rats given the tobacco smoke condensate, and their controls, showed severe changes in the delicate alveolar tissue and some hyperplasia of the bronchiolar epithelium. There was also a great deal of infiltration by inflammatory cells and macrophages with abscesses developing particularly in the rats treated with the condensate. However, none of the rats showed metaplastic or malignant changes of the lung tissues. Mammary gland tumours were found at 74, 95 and 104 weeks in three rats treated with condensate and in

TABLE III.—*Intratracheal Administration to Rats*

Substance administered	Dose (twice weekly unless stated)	Weeks administered	Appearance of lung tumours (weeks)	Time of deaths (weeks)	Other tumours (time in weeks)
Oil	(30 μl.)	30	—	62, 68, 72, 79, 80	—
β-Propiolactone in oil (30 μl.)	0·3 mg.	30	72, Squamous carcinoma of bronchii	12, 67, 72, 78, 89 and 100	—
Penicillic acid in oil (30 μl.)	0·3 mg.	30	—	13, 16, 72, 85, 86, 92	—
Aflatoxins in oil (30 μl.)	0·3 mg.	30	37, Tracheal squamous carcinoma, 52, ditto, 62, ditto.		Hepatoma and cholangioma (49), Hepatoma, renal adenoma, carcinoma of pylorus (49), Hepatoma (52), Hepatoma (62).
Methylproto-anemonin in oil (30 μl.)	0·6 mg.	30	—	10, 63, 100, 100, 100, 100	—
Benzopyrene in oil (30 μl.)	0·3 mg.	—	—	8, 25, 47, 100, 100	—
Controls without oil	—	—	—	16, 16, 28, 88, 99, 102	Thymoma (88) Mammary tumour (99)
Neutral fraction of cigarette smoke condensate	15–30 μl. once weekly	52	—	23, 43, 63, 63, 74, 74, 84, 101, 104, 104	Mammary tumour (74)
Neutral fraction of cigarette smoke conden-sate	15–30 μl. 3 times weekly	52	—	23, 39, 51, 64, 88, 95, 101, 104, 104, 104	Mammary tumours (95 and 104) Uterine tumour (104)

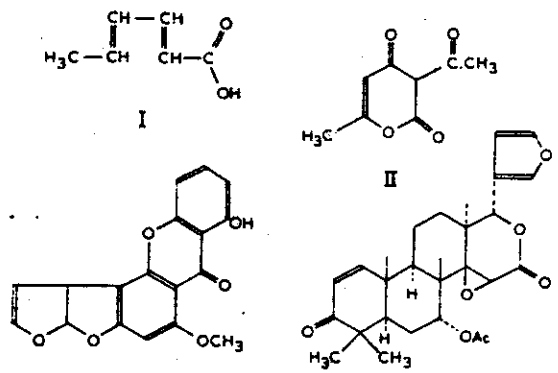
one of their controls at 99 weeks. There was also a tumour of the uterus at 104 weeks present in one rat treated with the tobacco tar, and a thymoma in one of the control rats which died at 88 weeks.

The aflatoxins, administered intratracheally, were effective carcinogens and produced a variety of tumours. In the trachea of 3 rats, invading squamous carcinomas (Fig. 3 and 4) developed eventually killing the animals by occluding the air passage at 37, 52 and 62 weeks respectively. The latter two animals also suffered from liver cancers (Fig. 1), as also did two other rats which died at 49 weeks. One of these also had a renal adenoma and a globular growth on the pyloric portion of the intestine which has been identified as a carcinoma (Fig. 2). β-Propiolactone by the same route induced a lung cancer in one of the rats. This tumour (Fig. 5 and 6) involved only one lobe of the lung and was an advanced, keratinising squamous cell carcinoma which killed the animal at 72 weeks. Three other rats treated intratracheally with β-propiolactone survived to 78, 89 and 100 weeks but without developing any tumours.

DISCUSSION

Previously (Dickens and Jones, 1965) 24 rats had been given repeated subcutaneous injections of arachis oil without developing tumours at the injection site although they had been observed for periods of up to 108 weeks. In the current

control experiment (where oil from the same importer, but a different batch, was used) the appearance of a tumour resembling a fibrosarcoma, though not with histological characteristics of malignancy, following repeated injections of the oil gives an overall incidence of one tumour in a total of 28 rats. This is a somewhat lower incidence than in similar control experiments with the same oil that we have carried out with a random bred strain of albino mice and indicates the suitability of the rat as a test animal.



Aflatoxin by the three routes of administration employed has shown itself to be a most effective carcinogen, though the dose requirement by different routes was not the same. Following subcutaneous injection twice weekly in 2 μg. doses a total of 166 μg. of the aflatoxins had been administered by the time the

EXPLANATION OF PLATES

FIG. 1. Mixed liver tumour in a rat given 300 μg. aflatoxins ($B_1 + G_1$) intratracheally twice-weekly for 30 weeks. The liver tumour was found at death 19 weeks after the last administration of aflatoxins. × 100.

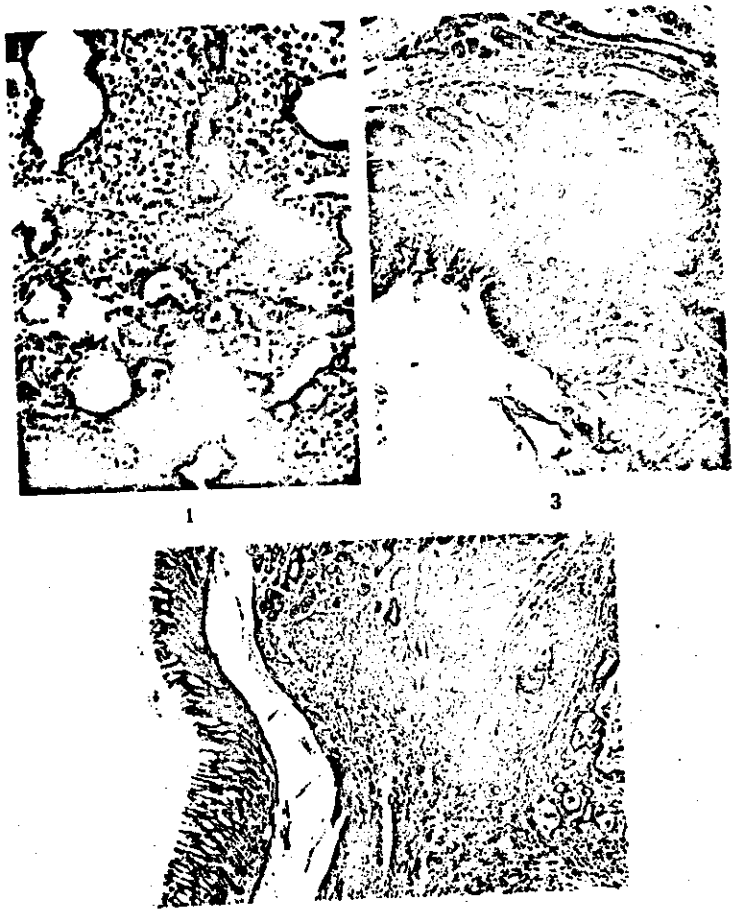
FIG. 2.—Adenocarcinoma of the intestine found in a rat which died 19 weeks after 30 weeks of treatment with 300 μg. aflatoxins ($B_1 + G_1$) intratracheally twice-weekly. Normal mucosa to the left of the picture, invading adenocarcinoma to the right. × 23.

FIG. 3. Squamous carcinoma of the tracheal epithelium of a rat invading the tracheal wall between the cartilaginous rings. This tumour was seen in a rat treated with twice weekly intratracheal doses of aflatoxins ($B_1 + G_1$, 300 μg.) for 30 weeks when it died 32 weeks later. × 23.

FIG. 4.—Squamous metaplasia and carcinoma in a tumour of the tracheal epithelium of a rat treated intratracheally with aflatoxins ($B_1 + G_1$, 300 μg.) × 100.

FIG. 5.—Keratinising squamous carcinoma of rat lung found at death in an animal 42 weeks after cessation of 30 weeks of intratracheal administration of 300 μg. β-propiolactone twice weekly. × 23.

FIG. 6.—Higher magnification of one area of the lung carcinoma shown in Fig. 5. × 100.



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first tumour was seen at 44 weeks. The total amount ingested continuously with the drinking water by the time tumours appeared in rats on the lower concentration was 2·2 mg., and at the higher concentration 13·6 mg. This result indicated that the subcutaneous test is at least ten times as sensitive as that by the oral route and for detection of carcinogenic activity its use is fully justified. Comparison of the amounts of pure aflatoxins administered in food by Barnes and Butler (1964) with those in the drinking water in the present experiments shows at least a rough correlation, though rather more was required in the drinking water. The induction of a lachrymal gland tumour in one of the rats given aflatoxins in their drink is also striking since Butler and Barnes (1963) obtained a similar tumour in a rat in their feeding experiments, using infected groundnuts suggesting that one excretion route for aflatoxin is via the secretion of this gland.

Sorbic acid has not previously been shown to be carcinogenic. It is an open-chain carboxylic acid with conjugate double bonds also conjugated with the terminal carboxyl, and has been shown to react appreciably with cysteine without acid production (Dickens and Cooke, 1965). When injected into rats it has given rise to a significant number of sarcomas at the sites of injection and on histological grounds these tumours are malignant. Sorbic acid, as the potassium salt, is widely used as a fungistatic agent added to a range of food products and is therefore a substance ingested by man. It is most important for this reason to determine whether it is capable of exerting a carcinogenic action when taken orally. This was attempted in rats by allowing the animals to drink a solution of sorbic acid neutralised with sodium bicarbonate. A total of approximately 2 g. sorbic acid was ingested by one rat in this experiment. Unfortunately, the other rats did not survive long enough to provide a clear answer and this experiment must be repeated, although the present result was negative.

Dehydroacetic acid, a lactone containing a carbonyl group conjugated with the cyclic $\gamma\delta$-double bond has also given a convincing number of malignant sarcomas when the free substance was injected in oil into rats. This substance is also used as an antifungal agent in the form of the sparingly soluble magnesium salt, e.g. for incorporation into the corks of containers of materials used for human consumption. In its reaction with cysteine (Dickens and Cooke, 1965) it closely resembled sorbic acid. This acid was also brought into solution in water by neutralising with sodium bicarbonate and given to rats to drink without causing the development of any tumours, although there were survivors at 103 weeks that had ingested approximately 2 g. during the experiment. Absence of carcinogenic activity was also reported by Spencer, Rowe and McCollister (1950) in experiments where dehydroacetic acid was incorporated in the diet of rats at concentrations of 0·02–0·10%.

If the same subcutaneous to oral dose-ratio applies to these acids as was calculated for aflatoxins it might be found necessary to administer still larger total doses to produce tumours of rat livers.

Sterigmatocystin, a difurano-xanthone derivative resembling in chemical structure the difurano-coumarin type present in the aflatoxins, is a metabolite of the mould *Aspergillus versicolor*, whereas the aflatoxins are produced by *Aspergillus flavus*. Although only 0·5 mg. of this substance was injected twice weekly and, owing to lack of material, injections had to be discontinued after only 24 weeks, 3 local sarcomas were induced with a delay of 47 weeks, as well as liver tumours in two rats. In contrast, not even large doses of the aflatoxins given

subcutaneously produced liver tumours, though they were so much more potent in their local effect. The difference in chemical structure is also related to greatly reduced, but not complete absence of carcinogenic activity in sterigmatocystin 0·5 mg doses of which show comparable activity with 2 μg. doses of aflatoxin as studied by Dickens and Jones (1963*b*, 1965).

Gedunin was only very weakly active or non-carcinogenic, though because of its chemical structure it was selected for testing as a possible carcinogen. It is a polycyclic lactone containing an epoxide group which has been isolated from African hardwoods of the "Mahogany" type (*Entandrophragma angolense*) (Bevan, Hassall, Nwagi and Taylor, 1962). The only sarcoma to develop was seen at 103 weeks and did not exhibit the proliferative activity and abnormal cell forms that indicate malignancy. Several of the rats injected with gedunin had earlier developed hard subcutaneous masses but these proved to be fibroblastic masses of normal reaction tissue. The only other abnormality was a carcinoma of the bladder seen in a rat killed because of an infected abscess at the injection site at 67 weeks.

Parasorbic acid, previously shown to induce tumours readily when injected in oil (Dickens and Jones, 1963*a*) was also supplied to two groups of rats to drink in water, without carcinogenic effect. Rats on the highest concentration received a total oral dose of about 200 mg. compared with an effective total dose subcutaneously of 25 mg.

The difficulties inherent in producing lung cancer in experimental animals with pure carcinogens are emphasised in our intratracheal experiments, where a number of substances including benzopyrene which have been shown to be carcinogenic by other routes, have proved to be ineffective. This is in agreement with the findings of many other workers who have failed to produce lung tumours in experimental animals. Successful tumour induction has required additional factors such as mechanical damage to the lung tissue (Blacklock, 1957), or adjuvants with the carcinogen, e.g. Tween 60 (Della Porter, Kolb and Shubik, 1958), carbon black (Steiner, 1954; Shabad, 1962) or finely divided ferric oxide (Saffiotti, Cefis and Kolb, 1964). That it is possible to induce tumours of the lung by pure substances is shown by the induction in the present work of a bronchogenic tumour with intratracheally administered β-propiolactone. The possibility that carcinogens of the reactive lactone type could be involved in human lung cancer requires investigation. Substances such as aesculin and scopoletin have been identified in tobacco smoke and from a consideration of their chemical structure would appear to be suitable candidates: tests by subcutaneous injection on these two compounds are in progress. Attempts to induce lung tumours in rats by the intratracheally administered neutral fraction of cigarette smoke condensate have given negative results in our experiments, in spite of the massive dosage. The possible role of co-carcinogens present in whole tobacco smoke has, of course, to be considered in relation to this negative finding.

The results with intratracheally administered aflatoxin are interesting, particularly because of the wide variety of tumours which have resulted by this route of administration. The aflatoxins are metabolites of moulds growing on vegetable material and the possibility needs to be explored that such contamination might be a source of carcinogens in some tobaccos. Inhalation of such mould products in dust by workers exposed e.g. to ground nuts infected with *Aspergillus flavus* may also constitute a possible cancer hazard

SUMMARY

1. Four further compounds, related to our previous series of carcinogenic lactones, have been tested for carcinogenic activity in the rat.

2. Two fungistatic agents used as preservatives in the foodstuff industry—sorbic acid and dehydroacetic acid—have been shown to be actively tumorigenic after their repeated subcutaneous injection in oil.

3. Sterigmatocystin, a metabolic product of the mould *Aspergillus versicolor*, which is chemically related to aflatoxin, was also definitely carcinogenic in similar tests, though with only about one-two hundred and fiftieth of the activity of aflatoxin. It produced not only local sarcomas but also liver tumours. Gedunin, a complex steroid-like epoxide present in tropical hardwoods, appeared to be only weakly carcinogenic, but one animal had numerous urinary stones associated with a carcinoma of the bladder wall.

4. Administration in the drinking water continuously for many weeks did not produce significant numbers of tumours with solutions of the following substances, which we have shown to be carcinogenic by the subcutaneous route: parasorbic acid, sorbic acid, dehydroacetic acid. On the other hand, aflatoxin given in the drinking water produced hepatocellular tumours in all rats receiving 100 μg./100 ml. water, but in only one of 6 rats given 10 μg. aflatoxin/100 ml. drinking water. The effective total oral dose was at least 10 times greater than the total subcutaneous dose previously found by us.

5. Intratracheal intubation twice weekly by micro-syringe into groups of rats gave no tumours with the known carcinogens penicillic acid, methyl protoanemonin or benzopyrene (0·3-0·6 mg. per dose twice weekly in 30 μl. oil for 30 weeks). A similar negative finding was observed in the lungs of rats after the intubation of undiluted cigarette smoke condensate (neutral fraction; 15-30 μl. doses, once or thrice weekly for 52 weeks) even in those surviving 100 weeks; some mammary tumours occurred in this series.

6. In contrast to the above, the same doses (0·3 mg.) of β-propiolactone induced a lung cancer (squamous carcinoma) in one survivor. Numerous carcinomata, including those of the trachea, liver, kidney and intestine, appeared in the rats intubated with aflatoxin intratracheally. The remarkable differences of sensitivity of lung tissue to carcinogenesis by certain chemical agents which are quite potent elsewhere in the body is clearly brought out by these experiments.

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REFERENCES

Barnes, J. M. and Butler, W. H.—(1964) *Nature, Lond.*, **202**, 1016.

Bevan, C. W. L., Hassall, T. G., Nwagi, M. N. and Taylor, D. A. H.—(1962) *J. chem. Soc.*, 768.

BLACKLOCK, J. W. S.—(1957) *Br. J. Cancer*, **11**, 181.
BULLOCK, E., ROBERTS, J. C. AND UNDERWOOD, J. G.—(1962) *J. chem. Soc.*, 4179.
BUTLER, W. H. AND BARNES, J. M.—(1963) *Br. J. Cancer*, **17**, 699.
DAVIES, J. E., KIRKALDY, D. AND ROBERTS, J. C.—(1960) *J. chem. Soc.*, 2169.
DELLA PORTER, G., KOLB, L. H. AND SHUBIK, P.—(1958) *Cancer Res.*, **18**, 592.
DICKENS, F.—(1963) Alkylierend wirkende Verbindungen: 1st. Conf. on N-Nitroso Compounds and Lactones, Hamburg. (Wissenschaftliche Forschungsstelle der Cigarettenindustrie), pp. 9-10.
DICKENS, F. AND COOKE, JUDITH.—(1965) *Br. J. Cancer*, **19**, 404.
DICKENS, F. AND JONES, H. E. H.—(1961) *Br. J. Cancer*, **15**, 85.
DICKENS, F. AND JONES, H. E. H.—(1963*a*) *Br. J. Cancer*, **17**, 100.
DICKENS, F. AND JONES, H. E. H.—(1963*b*) *Br. J. Cancer*, **17**, 691.
DICKENS, F. AND JONES, H. E. H.—(1965) *Br. J. Cancer*, **19**, 392.
LITTLEWOOD, G. AND PLATTS, T. L.—(1953) *J. Anim. Techns Ass.*, **4**, 53.
ROBERTS, J. C. AND UNDERWOOD, J. G.—(1962) *J. chem. Soc.*, 2060.
SAFFIOTTI, U., CEFIS, F. AND KOLB, L. H.—(1964) *Proc. Am. Ass. Cancer Res.*, **5**, 55.
SHABAD, L. M.—(1962) *J. natn. Cancer Inst.*, **28**, 1305.
SPENCER, H. C., ROWE, V. K. AND MCCOLLISTER, D. D.—(1950) *J. Pharmac. exp. Ther.*, **99**, 57.
STEINER, P.—(1954) *Cancer Res.*, **14**, 103.

TEST FOR CARCINOGENESIS OF CIGARETTE TOBACCO SMOKE CONDENSATE USING YOUNG STRONG A AND C57 BL MICE

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THE work here was designed on the basis of a previous experiment (Flaks, 1965), in which it was shown that Strong A mice are highly susceptible to the induction of adenoma of the lung, following the administration of 30 μg. of 9,10-dimethyl-1,2-benzanthracene (DMBA) to young animals.

In the present experiment, the test substances, denicotinized whole cigarette tobacco smoke condensate and its neutral fraction were tested.

The method for testing substances for carcinogenicity by subcutaneous injection into new-born mice, has been reported by Pietra, Spencer and Shubik (1959), Pietra, Rappaport and Shubik (1961), Stich (1960) and Roe, Rowson and Salaman (1961) using DMBA. Kelly and O'Gara (1961) and O'Gara, Kelly and Mantel (1962) reported favourable results with 1,2 : 5,6-dibenzanthracene and 20-methylcholanthrene. Roe, Mitchley and Walters (1963) tested 1,2-benzanthracene, 2-naphthylamine, 2-naphthylhydroxylamine and ethyl methane sulphonate and Grant and Roe (1963) investigated the interaction of phenanthrene with benzopyrene in carcinogenesis using the same technique.

Extensive work has been performed on the carcinogenic activity of tobacco smoke condensates: the review by Wynder and Hoffmann (1964) is cited.

MATERIALS AND METHODS

The animals used were Strong A and C57Bl strains of mice, bred in this laboratory by selective brother-sister mating.

The test substances used were whole denicotinized cigarette tobacco smoke condensate and its neutral fraction. Both were prepared by the Tobacco Research Council, using the smoking technique recommended by the Tobacco Manufacturers Standing Committee (Bentley and Burgan, 1961). The removal of nicotine was necessary, owing to its high toxicity.

Suspensions of condensates in 3% aqueous gelatine were prepared by adding acetone solutions of condensates to the gelatine at 56° C. The acetone was removed by passing a nitrogen stream through the suspension.

Treated and solvent-control animals were placed in groups of sixty mice, containing equal number of males and females. For each test substance and each strain of mice, new-born (up to 12 hours old), seven- and fourteen-days-old groups were used.

Test animals of all groups received one subcutaneous injection, in the interscapular region, of 300 μg. of test substance in 15 μl. of 3% aqueous gelatine. Solvent controls received 15 μl. of 3% aqueous gelatine only. Animals without treatment were also included.

potency in the rabbit. Whatever the explanation of this unexpected result with electrolysin, it seems noteworthy that this is still another example of a biological substance exhibiting wide variations in permeability effects according to the species of test animal[9].

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[1] Lewis, G. P., *Ann. N.Y. Acad. Sci.*, **104**, 236 (1963).
[2] Wilhelm, D. L., *Pharmacol. Rev.*, **14**, 251 (1962).
[3] Carr, Jean, and Wilhelm, D. L., *Austral. J. Exp. Biol. and Med. Sci.*, **42**, 511 (1964).
[4] Sparrow, Elizabeth M., and Wilhelm, D. L., *J. Physiol.*, **137**, 51 (1957).
[5] Konzett, H., and Stürmer, E., *Brit. J. Pharmacol.*, **15**, 544 (1960).
[6] Lewis, G. P., *Nature*, **192**, 596 (1961).
[7] Webster, Marion E., and Pierce, J. V., *Ann. N.Y. Acad. Sci.*, **104**, [illegible] (1963).
[8] Schachter, M., *Ann. N.Y. Acad. Sci.*, **104**, 108 (1963).
[9] Margolis, J., and Bishop, Elizabeth A., *Austral. J. Exp. Biol. and Med. Sci.*, **41**, 293 (1963).
[10] Rubin, B., Waugh, Margaret H., Laffan, R. J., O'Keeve, Erika, and Craver, B. N., *Proc. Soc. Exp. Biol.*, **113**, 328 (1963).
[11] Spector, W. G., *J. Path. Bact.*, **63**, 93 (1951).
[12] Woolley, D. W., and Merrifield, R. B., *Ann. N.Y. Acad. Sci.*, **104**, 161 (1963).
[13] Erspamer, V., and Anastasi, A., *Experientia*, **18**, 58 (1962).
[14] Miles, A. A., and Miles, E. M., *J. Physiol.*, **118**, 228 (1952).
[15] Wilhelm, D. L., Mill, P. J., Sparrow, Elizabeth M., Mackay, Margaret E., and Miles, A. A., *Brit. J. Exp. Path.*, **39**, 228 (1958).
[16] Clausen, D. F., and Lifson, N., *Proc. Soc. Exp. Biol.*, **91**, 11 (1956).
[17] Rawson, Ruth A., *Amer. J. Physiol.*, **138**, 708 (1942-3).
[18] Ascheim, E., *Nature*, **201**, 1291 (1964).
[19] Judah, J. D., and Willoughby, D. A., *J. Path. Bact.*, **83**, 567 (1962).
[20] Spector, W. G., and Willoughby, D. A., *Nature*, **196**, 1101 (1962).

A SIMPLE BIOASSAY FOR ANTIOXIDANTS BASED ON PROTECTION OF *Tetrahymena pyriformis* FROM THE PHOTODYNAMIC TOXICITY OF BENZO(*a*)PYRENE

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ANTIOXIDANTS are widely used in food, as well as in other natural or synthetic products such as petroleum, rubber and paints, to inhibit autoxidative deterioration. This subject has been well reviewed in recent publications[1,2]. While a voluminous literature exists on chemical tests for antioxidants, biological data on their *in vivo* activity, perforce, have been restricted to some studies on vitamin E biopotency[3]. The present article describes a simple and rapid biological method for the *in vivo* characterization and assay of antioxidant activity based on the inhibition of the oxygen-dependent phenomenon, photodynamic injury[4-7]. This approach was stimulated by the finding that vitamin E protects a ciliated protozoan against the photodynamic effects of a polycyclic photosensitizer[6,7].

The compounds tested are conventionally grouped, as in Table 1. In general, *primary antioxidants* are considered to function as free-radical acceptors; *secondary antioxidants* act synergistically with primary antioxidants; and *ultra-violet absorbers* block the catalytic effect of ultra-violet and visible light. The *miscellaneous group*, comprised largely of reducing agents with free —SH groups, includes classical radioprotective agents not generally regarded as antioxidants.

From stock solutions of compounds in acetone or water at 0·1 or 0·01 g/ml., serial log dilutions of aqueous suspensions were freshly prepared from 3,000 to 0·3 μg/ml. The dilution range was appropriately extended, occasionally to as low as 3×10^{-10} μg/ml., for compounds with high activity. Aqueous suspensions of benzo(*a*)pyrene (B*a*P) at 0·1 μg/ml., freshly prepared from stock acetone solutions of 100 μg/ml., were used as the standard photosensitizing agent. Cultures of *Tetrahymena pyriformis* var 1, mating type II, were maintained axenically in the dark at 28° C in medium containing 1·5 per cent w/v 'Proteose®' peptone and 0·15 per cent w/v yeast extract at *p*H 6·8. Suspensions from log-phase cultures, diluted in medium to contain approximately 30 cells, were mixed on single 15 × 4 mm plastic depression slides with aliquots of B*a*P and of individual concentrations of antioxidants. These slides, together with a minimum of two antioxidant-free controls, were incubated at 28° C in dark moist chambers for a minimum of 2 h. Two wells were then irradiated simultaneously with long-wave ultra-violet light, of 8,750 μW/cm² at 45 cm, filtered to transmit light from 310 to 400 mμ with a peak at 366 mμ, and observed directly with a binocular stereoscopic microscope at a magnification of × 10. The duration of irradiation required to produce lethality, as indicated by immobilization of 90 per cent of the motile ciliates (LT_{90}), was determined, with an arbitrary observation limit of 60 min. All compounds were tested a minimum of three times, using new stock solutions on at least two occasions.

Protection from photodynamic toxicity was manifest by prolongation of median LT_{90} over median control values (Table 2). Concentrations of compounds producing two-fold prolongation of LT_{90} values and dose-response slopes of those compounds were determined (Table 1 and Fig. 1). Approximately half the compounds tested (58/98) achieved such protection. α-Tocopherol was selected as the standard for apparent relative potency determinations (Table 1). For compounds yielding two-fold or greater protection, the possible role of light absorption was

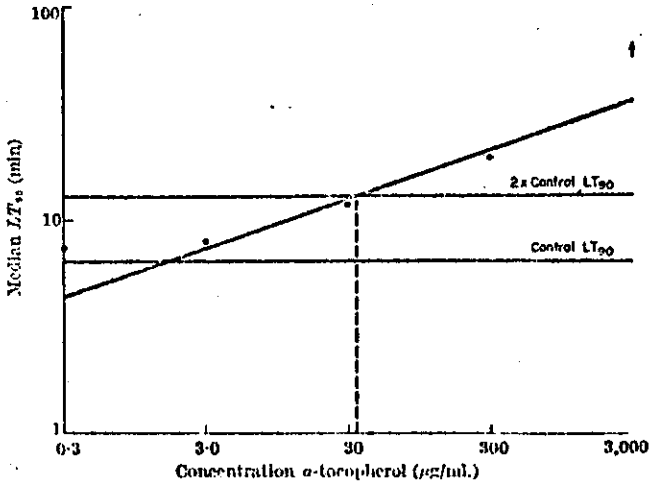

Fig. 1. Method for determining the concentration of α-tocopherol required to produce two-fold prolongation of control LT_{90} values. α-Tocopherol was used as the standard for relative potency determinations. At 3,000 μg/ml., LT_{90} values exceeding the arbitrary observational limit of 60 min are indicated by the arrow

Table 1. EFFECTS OF ANTIOXIDANTS ON THE RESPONSE OF *T. pyriformis* TO PHOTODYNAMIC INJURY BY BENZO(*a*)PYRENE

Compounds*	Inhibition by antioxidants of photodynamic activity of 0·1 μg/ml. of benzo(*a*)pyrene			
	Conc. (μg/ml.) required for 2-fold prolongation of LT_{50} values†	Apparent antioxidant potency relative to α-tocopherol	Dose-response slopes (log/log)	% Photodynamic inhibition attributable to light absorption‡
PRIMARY ANTIOXIDANTS				
Phenolic Compounds				
Phenols				
1 Hydroquinone[1]	> 30	< 1·1	—	—
2 Salicylic acid[1]	> 300	< 0·1	—	—
3 *p*-Aminosalicylic acid[1]	> 300	< 0·1	—	—
4 2,5-Dihydroxybenzoic acid ('Gentisic acid')[2]	> 300	< 0·1	—	—
5 3,3',4',5,7-Pentahydroxyflavone ('Quercetin')[1]	12	2·8	0·32	46
6 Methyl-*p*-hydroxybenzoate ('Methylparaben')[1]	> 300	< 0·1	—	—
7 Ethyl-*p*-hydroxybenzoate ('Ethylparaben')[1]	> 30	< 1·1	—	—
8 Propyl-*p*-hydroxybenzoate ('Propylparaben')[1]	> 30	< 1·1	—	—
9 *n*-Propylgallate[2]	> 300	< 0·1	—	—
10 2,4,5-Trihydroxybutyrophenone[1]	12	2·8	0·24	0
'Hindered' Phenols				
11 Mixture of 2 and 3-*tert*.butyl-4-methoxyphenol (BHA)[1]	> 30	< 1·1	—	—
12 2,6-Di-(*tert*.)butyl-*p*-cresol (BHT)[1]	> 3,000	< 0·01	—	—
13 *tert*.Butylhydroquinone[1]	> 300	< 0·1	—	—
14 2,5-*Bis*(1,1-dimethylpropyl)hydroquinone (*tert*.amylhydroquinone)[1]	75	0·4	0·10	0
15 Undefined mixture of guaiacolic acids (gum guaiac)[1]	2·1	16	0·12	0
16 4,4'-(2,3-Dimethylteramethylene)dipyrocatechol (NDGA)[2]	$1·6 \times 10^{-3}$	$2·1 \times 10^{3}$	0·14	0
17 O,O-Di-*n*-octadecyl-3,5-di-*tert*.butyl-4-hydroxybenzylphosphonate[1]	15	2·2	0·31	0
18 2,4-*Bis*-(*n*-octylthio)-6-(4-hydroxy-3,5-di-*tert*.butylanilino)-1,3,5-triazine[1]	11	3·1	0·26	0
19 2,4-*Bis*-(*n*-octylthioethylthio)-6-(3,5-di-*tert*.butyl-4-hydroxyanilino)-1,3,5-triazine[1]	45	0·7	0·27	0
20 2,4-*Bis*(*n*-octylthioethylthio)-6-(4-hydroxy-3,5-di-*tert*.butylphenoxy)-1,3,5-triazine[1]	5·4	0·1	0·22	0
21 2,4-*Bis*(4-hydroxy-3,5-di-*tert*.butylphenoxy)-6-(*n*-octylthio)-1,3,5-triazine[1]	41	0·8	0·26	0
22 4-Hydroxymethyl-2,6-di-*tert*.butylphenol ('Ionox 100')[1]	> 3,000	< 0·01	—	—
23 2-(3,5-Di-*tert*.butyl-4-hydroxybenzylthio)benzothiazole ('Ionox 115')[1]	150	0·2	0·34	0
24 3,5-Di-*tert*.butyl-4-hydroxybenzyl ether ('Ionox 201')[1]	68	0·5	0·31	0
25 1,4-*Bis*-(3,5-di-*tert*.butyl-4-hydroxybenzyl)tetramethylbenzene ('Ionox 242')[1]	300	0·09	0·31	0
26 2,4,6-*Tris*-(3,5-di-*tert*.butyl-4-hydroxybenzyl)phenol ('Ionox 312')[1]	750	0·04	0·28	0
27 1,3,5-Trimethyl-2,4,6-*tris*(3,5-di-*tert*.butyl-4-hydroxybenzyl)benzene ('Ionox 330')[1]	210	0·2	0·29	0
Tocopherols				
28 α-Tocopherol[3]	33	1·0	0·33	0
29 β-Tocopherol[3]	13	2·5	0·27	0
30 γ-Tocopherol[3]	54	0·6	0·28	0
31 δ-Tocopherol[3]	39	0·9	0·27	0
32 5,5'-Bi-α-tocopherol[3]	51	0·7	0·33	0
33 D-α-Tocopheryl acid succinate[3]	20	1·7	0·33	0
34 D-α-Tocopheryl acetate[3]	60	0·6	0·33	0
35 D-α-Tocopheryl polyethylene glycolsuccinate[3]	3·6	0·2	0·32	0
Amines				
Aliphatic				
36 2,2',2"-Nitrilotriethanol ('Triethanolamine')[1]	> 3,000	< 0·01	—	—
37 Hydroxylamine hydrochloride[1]	> 300	< 0·1	—	—
Aromatic				
38 Di-*o*-tolylguanidine salt of dicatechol borate ('Permalux')[2]	27	1·2	0·12	0
39 Diphenylamine[1]	> 300	< 0·1	—	—
40 4,4'-Diphenyl-*p*-phenylenediamine (DPPD)[1]	75	0·4	0·24	0
41 Mixture containing *n*-phenyl-α-naphthylamine ('Neozone *A*')[2]	> 30	< 1·1	—	—
42 Mixture containing *n*-phenyl-β-naphthylamine ('Neozone *B*')[1]	> 300	< 0·1	—	—
43 Mixture containing 4,4'-dimethoxydiphenylamine, DPPD and 'Neozone *D*' ('Thermoflex')[2]	4·2	7·9	0·22	0
44 *p*-Aminobenzenesulphonylguanidine ('Sulphaguanidine')[10]	> 3,000	< 0·01	—	—
Nitrogen heterocyclics				
45 Isonicotinic acid hydrazide ('Isoniazid')[2]	> 3,000	< 0·01	—	—
46 2,3 : 5,6-Dibenzo-1,4-thiazine (phenothiazine)[1]	> 3,000	< 0·01	—	—
47 3,7-*Bis*(dimethylamino)phenazathionium chloride (methylene blue)[1]	30	1·1	0·25	0
48 3-Amino-1H-1,2,4-triazole[2]	> 3,000	< 0·01	—	—
49 6-Ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline (EMQ)[11]	60	0·6	0·10	57
50 8-(4-Amino-1-methylbutylamino)-6-methoxyquinoline ('Primaquin')[12]	11	3·0	0·21	0
51 7-Chloro-4-(4-diethylamino-1-methylbutylamino)-3-methylquinoline ('Nivaquin C')[12]	75	0·4	0·20	75
52 7-Chloro-4-(4-diethylamino-1-methylbutylamino)quinoline (chloroquine diphosphate)[13]	> 3,000	< 0·01	—	—
53 7-Chloro-4-[4-(ethyl-(2-hydroxyethyl)-amino)-1-methylbutylamino-]-quinoline (hydroxychloroquine)[13]	> 3,000	< 0·01	—	—
Aminoazobenzenes				
54 2,2'-Azonaphthalene[14]	13	2·5	0·16	16
55 4-Aminoazobenzene[14]	$1·5 \times 10^{-3}$	$2·2 \times 10^{3}$	0·21	0
56 4-Methylaminoazobenzene[14]	$1·0 \times 10^{-4}$	$3·3 \times 10^{4}$	0·10	0
57 4-Ethylaminoazobenzene[14]	$4·8 \times 10^{-3}$	$6·9 \times 10^{2}$	0·15	0
58 4-Dimethylaminoazobenzene ('Butter Yellow')[1]	$6·0 \times 10^{-5}$	$5·5 \times 10^{5}$	0·16	0
59 4-Diethylaminoazobenzene[14]	$3·1 \times 10^{-5}$	$1·1 \times 10^{6}$	0·11	0
60 4-Ethylbutylaminoazobenzene[14]	$5·3 \times 10^{-5}$	$6·2 \times 10^{5}$	0·11	0
61 2-Methyl-4-dimethylaminoazobenzene[14]	$1·2 \times 10^{-4}$	$2·8 \times 10^{5}$	0·11	0
62 2'-Methyl-4-dimethylaminoazobenzene[14]	$2·1 \times 10^{-4}$	$1·6 \times 10^{5}$	0·11	0
63 3-Methyl-4-dimethylaminoazobenzene[14]	$1·0 \times 10^{-3}$	$3·3 \times 10^{4}$	0·22	0
64 3'-Methyl-4-dimethylaminoazobenzene[14]	$2·1 \times 10^{-4}$	$1·6 \times 10^{5}$	0·15	0
65 4'-Methyl-4-dimethylaminoazobenzene[14]	$1·3 \times 10^{-4}$	$2·4 \times 10^{5}$	0·12	0
66 4'-Ethyl-4-dimethylaminoazobenzene[14]	$3·6 \times 10^{-4}$	$9·2 \times 10^{4}$	0·19	0
67 2',3-Dimethyl-4-aminoazobenzene[14]	$1·2 \times 10^{-4}$	$2·8 \times 10^{5}$	0·13	0
68 2-Methyl-4'-ethyl-4-dimethylaminoazobenzene[14]	$1·0 \times 10^{-4}$	$3·3 \times 10^{4}$	0·16	0

Table 1—*Continued*

Compounds*	Inhibition by antioxidants of photodynamic activity of 0·1 μg/ml. of benzo(*a*)pyrene			
	Conc. (μg/ml.) required for 2-fold prolongation of LT'_{90} values†	Apparent antioxidant potency relative to α-tocopherol	Dose-response slopes (log/log)	% Photodynamic inhibition attributable to light absorption‡
SECONDARY OR SYNERGISTIC ANTIOXIDANTS				
69 Boric acid[1]	> 3,000	< 0·01	—	—
70 Formic acid[1]	> 300	< 0·1	—	—
71 Benzoic acid[1]	> 300	< 0·1	—	—
72 Tartaric acid[1]	> 3,000	< 0·01	—	—
73 *Trans-trans*-2,4-hexadienoic acid (sorbic acid)[4]	> 300	< 0·1	—	—
74 Ascorbic acid[4]	> 300	< 0·1	—	—
75 Isoascorbic acid[1]	> 300	< 0·1	—	—
76 Sodium ascorbate[4]	> 3,000	< 0·01	—	—
77 Ascorbylpalmitate[4]	0·7	50	0·17	0
78 3,3'-Thiodipropionic acid[2]	> 300	< 0·1	—	—
79 Dilauryl thiodipropionate ('Mitban *F*')[5]	90	0·4	0·23	0
80 Lecithin[7]	$4·8 \times 10^{-3}$	600	0·18	0
81 Undefined mixture of wheat germ oil and lecithin complex ('Viobin' antioxidant)[14]	$2·4 \times 10^{-3}$	$1·4 \times 10^{3}$	0·14	0
MISCELLANEOUS				
Polyenes				
82 β-Carotene[6]	3	11	0·28	0
83 Xanthophyll[6]	9·0	3·7	0·28	0
84 Vitamin A[6]	6·0	5·5	0·26	0
Sulphur-containing Compounds				
85 Cysteine hydrochloride[1]	> 3,000	< 0·01	—	—
86 2,2-Diaminosiethylsulphide (cystamine)[15]	> 3,000	< 0·01	—	—
87 2-Mercaptoethylamine hydrochloride (cysteamine hydrochloride)[16]	900	0·04	0·10	0
88 2,3-Dimercaptopropanol (BAL)[15]	> 30	< 1·1	—	—
89 *S*-(2-Aminoethyl)isothiouronium bromide hydrobromide (AET)[15]	> 3,000	< 0·01	—	—
90 Tetraethylthiuramdisulphide ('Antabuse')[3]	$1·9 \times 10^{-3}$	$1·7 \times 10^{4}$	0·11	0
91 2-(*n*-Decylamino)ethanethiol[2]	> 30	< 1·1	—	—
ULTRA-VIOLET ABSORBERS				
92 2(2'-Hydroxy-5'-methylphenyl)benzotriazole ('Tinuvin *P*')[7]	150	0·2	0·21	100
93 2(2'-Hydroxy-5'*tert*.butylphenyl)benzotriazole[7]	81	0·4	0·23	100
94 *p*-Octylphenylsalicylate[1]	33	1·0	0·24	0
95 Hexamethylphosphoric triamide[1]	> 3,000	< 0·01	—	—
96 2,4-Dihydroxybenzophenone[1]	> 30	< 1·1	—	—
97 Resorcinol monobenzoate[1]	> 30	< 1·1	—	—
98 4-Dodecyloxy-2-hydroxybenzophenone[1]	12	2·8	0·25	35

* Source: [1]Eastman Chemical Products, Inc., Kingsport, Tenn.; [2]Aldrich Chemical Co., Milwaukee, Wis.; [3]Distillation Products, Rochester, N.Y.; [4]Weyerhauser Co., Tacoma, Wash.; [5]Nutritional Biochemicals Co., Cleveland, Ohio; [6]Will Corp., Rochester, N.Y.; [7]Geigy Industrial Chemicals, Ardsley, N.Y.; [8]Shell Chemical Co., New York, 20, N.Y.; [9]DuPont De Nemours and Co., Wilmington, Del.; [10]K and K Labs., Inc., Long Island, N.Y.; [11]Monsanto Chemical Co., St. Louis, Mo.; [12]Sterling Winthrop Research Inst., Rensselaer, N.Y.; [13]Dr. J. A. Miller, McArdle Memorial Laboratory, Madison, Wis.; [14]Viobin Corp., Monticello, Ill.; [15]Light and Co., Colnbrook, Bucks, England. We acknowledge generous gifts of samples from the following sources: [1, 3, 4, 7, 8, 9, 11, 12, 13].

† LT_{90} is the time in minutes required to immobilize 90 per cent of *T. pyriformis*. Where a doubling of the LT_{90} was not achieved at the highest concentration tested, this is indicated by the > sign. In such instances, assay slopes and absorption values could not be determined.

‡ The prolongation of LT_{90} values resulting from interposing solutions of the compounds in a cuvette between the light source and mixtures of cells and BaP is expressed as a percentage of the prolongation of LT_{90} values as determined when the same concentration of compound is tested directly with cells and BaP, in the absence of the cuvette, as in the regular assays.

investigated by interposing a cuvette, containing an acetone or water solution of the compound in a 1-mm light path, between the irradiation source and wells containing cells and BaP only; compounds were generally tested at the minimum concentration producing maximum protection in antioxidant assays. The resulting LT'_{90} values were compared with those of controls obtained with acetone or water in the cuvette.

The reproducibility of the assay is good (Table 2), although variation occurs on ageing of stock solutions, particularly with 'Butter Yellow' and 'Antabuse'. Protective activity increases progressively with concentration of antioxidant (Fig. 1). The assay reveals wide variations in potency, spanning a 10 log range, although this is sometimes limited by cytotoxicity at high concentrations. Dose-response slopes tend to be shallow, and also parallel in the case of close structural variants such as hindered phenols of the triazine series, 'Ionox' compounds, or tocopherols (Table 1).

Table 2. PROTECTION OF *T. pyriformis* FROM PHOTODYNAMIC TOXICITY OF BENZO(*a*)PYRENE BY VARYING CONCENTRATIONS OF α-TOCOPHEROL

Individual experiments	Time to 90 per cent lethality (min), with 0·1 μg benzo(*a*)pyrene, as photosensitizer, at specified concentrations, μg/ml., of α-tocopherol					
	3,000	300	30	3·0	0·3	0*
1	> 60	18	12	6·5	5·5	5·5
2	> 60	21	15	8	7·5	6
3	> 60	20	11	7·5	7·5	7
4	> 60	25	17	10	8·5	7
5	> 60	15	11	10	7	6·5
Medians	> 60	20	12	8	7·5	6·5

* Antioxidant-free control times represent means of 2–4 determinations in individual experiments.

Comparable potencies obtain for the four tocopherol isomers and acetate or succinate esters of the α-isomer; *in vivo* hydrolysis of the esters to the presumed active free tocopherol is possible. The importance of solubility is suggested by the enhanced activity of the water-soluble glycol-succinate ester, although on a weight basis it contains only about 25 per cent α-tocopherol. Although the dimeric form of tocopherol has relatively feeble vitamin E activity on gestation–resorption bioassay[8], it protects against photodynamic injury in a similar manner to the monomers. Antioxidants with reported vitamin E biopotency, such as methylene blue, EMQ, DPPD, NDGA, and 'Antabuse', are all active in the photodynamic bioassay; the latter two compounds in particular showing high activity. Phenols and the simple hindered phenols, BHA, BHT, and 'Ionox 100', are generally inactive in contrast to more complex phenols, such as the other

'Ionox' antioxidants and the substituted triazines. The antioxidant activity of the aminoazobenzenes, previously described in other systems[9], was generally found to be extremely high (Table 1), and 'Butter Yellow', for example, is almost one million times more potent than α-tocopherol. As it is unlikely that differences of such magnitude are explicable in terms of free radical acceptance alone, biological factors such as cell uptake, and selective localization or complexing, are probably involved. There is no apparent correlation between the hepatocarcinogenic and antioxidant activities of the aminoazobenzenes. (Hepatocarcinogenic potencies of aminoazobenzenes in rats: + + + +, Nos. 64, 66, 68; + + +, Nos. 56, 58, 60; + +, No. 62; +, Nos. 63, 65, 67; 0, Nos. 55, 57, 59, 61. No. 54 is hepatocarcinogenic in the mouse[10].) Secondary antioxidants, palmitic and dilauryl esters of ascorbic, and 3,3'-thiodipropionic acids, respectively, showed activity in contrast to the acids. Since commercial samples of lecithin may be contaminated with tocopherol, the activities of lecithin and 'Viobin' observed here may reflect antioxidant synergism between lecithin and varying amounts of tocopherol. Apart from the very low potency of cysteamine, radioprotective agents were inactive. The cuvette experiments (Table 1) indicate the role of absorption in the demonstrated activity of primary antioxidants, such as 'Quercetin' and EMQ. Protection by some industrial ultra-violet absorbers, such as 'Tinuvin P', may be due to low transmittance at the 360 mμ region; however, the effects of others are not explicable in this way.

The present assay provides a sensitive biological measure of particular *in vivo* effects of certain antioxidants, but its general applicability and specificity cannot, as yet, be defined. Present experiments[11] indicate that, in general, antioxidants yield similar responses in the photodynamic bioassay irrespective of which particular photosensitizing agent, carcinogenic or otherwise[12,13], is used and also that antioxidant activity is inversely related to the concentration of the challenge photosensitizer. Attempts are being made to define the quantitative interaction between antioxidant and photosensitizer, *inter alia*, by determination of inhibition indices, and also the role of cell-uptake in determining activity[11]. The results of these *in vivo* assays are further being contrasted with those obtained by *in vitro* photodynamic bioassays, using isolated rat liver mitochondria[11], and by previously described *in vitro* haemolysis tests[14]. In addition, the effects of selected antioxidants on the response of mice to a polycyclic carcinogen are being investigated[11]. These and other aspects of this work will shortly be reported.

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[1] *Autoxidation and Antioxidants*, 1 and 2, edit. by Lundberg, W. O. (Interscience Publishers, New York, 1961).
[2] *Symposium on Foods: Lipids and Their Oxidation*, edit. by Schultz, H. W., Day, E. A., and Sinnhuber, R. O. (The Avi Publishing Co., Westport, Conn, 1962).
[3] Bieri, J. G., in *Progress in the Chemistry of Fats and Other Lipids*, 7 (2), edit. by Holman, R. T. (Pergamon Press, Macmillan Co., New York, 1964).
[4] Blum, H. F., *Physiol. Rev.*, 12, 23 (1932).
[5] Santamaria, L., *Rec. Contrib. Cancer Res. Italy*, 1, 167 (1960).
[6] Epstein, S. S., and Burroughs, M., *Nature*, 193, 337 (1962).
[7] Epstein, S. S., Burroughs, M., and Small, M., *Cancer Res.*, 23, 35 (1963).
[8] Nelan, D. R., and Robeson, C. D., *J. Amer. Chem. Soc.*, 84, 2963 (1962).
[9] Rusch, H. P., and Miller, J. A., *Proc. Soc. Exp. Biol. and Med.*, 67, 140 (1948).
[10] Miller, J. A. (personal communication).
[11] Epstein, S. S. (to be published).
[12] Epstein, S. S., Small, M., Falk, H., and Mantel, N., *Cancer Res.*, 24, 855 (1964).
[13] Epstein, S. S., Bulon, I., Koplan, J., Small, M., and Mantel, N., *Nature*, 204, 750 (1964).
[14] Rose, C. S., and Gyorgy, P., *Amer. J. Physiol.*, 168, 414 (1952).

THREE HAEMOGLOBINS K: WOOLWICH, AN ABNORMAL, CAMEROON AND IBADAN, TWO UNUSUAL VARIANTS OF HUMAN HAEMOGLOBIN A

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HAEMOGLOBIN K was first described by Cabannes and Bahr[1] and is a 'fast' variant of normal adult haemoglobin (haemoglobin A). It moves further towards the anode on electrophoresis at alkaline *p*H than haemoglobin A, but only just separates from it.

A haemoglobin K with its abnormality in the β-chain has been reported to occur in combination with haemoglobin S (ref. 2). The proportion of S : K was 3 : 2, and there was a mild haemoglobinopathy. The family in which this haemoglobin K was observed came from the West Indies and was of African ancestry. This haemoglobin K was first noted at Woolwich, England, and it will be denoted as K β Woolwich.

We have now found a second instance of the combination of a haemoglobin K with haemoglobin S. By hybridization with canine haemoglobin[3] this haemoglobin K could be shown to be a β-chain variant. Unlike haemoglobin K Woolwich, the proportion of this haemoglobin K to haemoglobin S was 3 : 1, similar to that of haemoglobin A in the sickle-cell trait (A + S). There was no haemoglobinopathy. Indeed, the carrier of this K + S combination was found in the course of routine screening of healthy blood donors at University College Hospital, Ibadan, Nigeria. The blood donor, a woman, came from Cameroon, and neither she herself nor her relatives could be examined further. This new haemoglobin K will be denoted as haemoglobin K β Cameroon (Figs. 1 and 2).

A third sample of haemoglobin K, this time in combination with haemoglobin A, was found in a Yoruba adolescent in Ibadan, in the course of an anthropological survey.

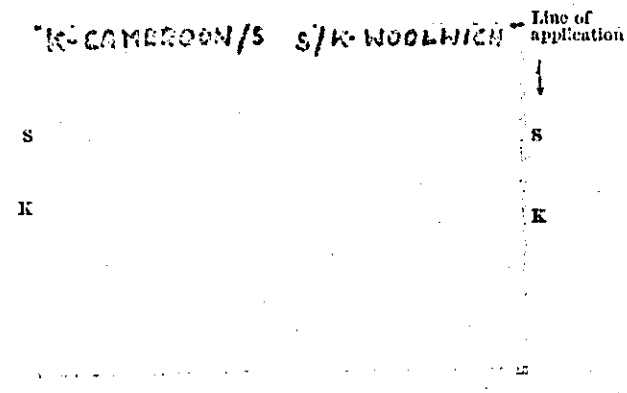

Fig. 1. Paper electrophoresis at *p*H 8·6 of haemoglobins K Cameroon + S (left) and S + K Woolwich (right). The proportion of K Cameroon to S is that found for A in sickle-cell trait (see Fig. 2)

an "olefinic hydrogen" ($\tau = 4.57$) and four methylene groups, two of which should be adjacent to carbonyl groups (signal groups centered at $\tau = 7.7$ and 8.3). Signals from the enolic proton and the carboxylic proton were not seen, presumably due to intermolecular exchange. These facts, as well as the red-violet colour produced with iron(III) chloride, indicate that the compound is the completely enolised 5,7-dioxononanic acid ("adipylacetone"). No evidence for the formation of cycloheptane-1,3-dione [17] in the hydrolysis has so far been obtained.

Triacetylmethane. The reaction was run with acetic anhydride in dichloroethane with boiling for 0.5 h. Isolation by continuous extraction with ether, extraction with sodium hydrogen carbonate, continuous ether extraction of the acidified solution and distillation. Triacetylmethane, b.p. 100°/20 mm, was obtained in 10 % yield. IR and NMR spectra were identical with those previously reported for triacetylmethane prepared by acetylation of acetylacetone.[11,18]

From other fractions acetylacetone (*ca.* 25 %) and a small amount of 2,6-dimethylpyrone (*ca.* 0.5 %) were isolated. The first may have been formed by hydrolysis of triacetylmethane, rearrangement of isopropenyl acetate or by mono-acylation.

Unsuccessful attempts. α,α-Dimethylglutaric acid rapidly evolves carbon monoxide on contact with aluminium chloride. An attempt to add equivalent amounts of the anhydride and isopropenyl acetate to a cold, stirred suspension of aluminium chloride in dichloroethane gave extensive CO elimination and the main product was the lactone of 4-hydroxyisovaleric acid (*ca.* 60 %, identified by b.p. 98—99°/20 mm and by IR and NMR spectra). Only traces of enolic material were obtained.

Reaction of tetrahydrophthalic anhydride (from maleic anhydride and butadiene) in dichloroethane with boiling 1 h gave a product which was isolated *via* sodium carbonate extraction and distillation (*ca.* 8 g. 40 %). Infrared spectra and colour reactions were very similar to those of 2-acetyl-3a,4,7,7a-tetrahydroindane-1,3-dione [3] (obtained from 2-acetylcyclopent-4-ene-1,3-dione and butadiene [3]). The oil did not crystallise even on seeding and presumably there had been partial rearrangement of the double bond under the vigorous reaction conditions.

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REFERENCES

1. Merényi, F. and Nilsson, M. *Acta Chem. Scand.* 17 (1963) 1801.
2. Nilsson, M. *Acta Chem. Scand.* 18 (1964) 441.
3. Nilsson, M. and Merényi, F. *Swedish Patent Application* 5285 (1963).
4. Hagemeyer, H. J. and Hull, D. C. *Ind. Eng. Chem.* 41 (1949) 2920.
5. Merényi, F. and Nilsson, M. *To be published.*
6. Berlinor, E. *Org. Reactions* 5 (1949) 229, 247.
7. Sieglitz, A. and Horn, O. *Chem. Ber.* 84 (1951) 607.
8. Hauser, C. R., Frostich, F. C. and Man, E. H. *J. Am. Chem. Soc.* 74 (1952) 3231.
9. Youssefyeh, R. D. *J. Am. Chem. Soc.* 85 (1963) 3901.
10. Stork, G., Brizzolara, A., Landesman, H., Smuszkowics, J. and Terell, R. *J. Am. Chem. Soc.* 85 (1963) 207.
11. Hauser, C. R., Swamer, F. W. and Adams, J. T. *Org. Reactions* 8 (1954) 59.
12. Forsén, S., Merényi, F. and Nilsson, M. *Acta Chem. Scand.* 18 (1964) 1208.
13. Boothe, J. H., Wilkinson, R. G., Kushner, S. and Williams, J. *J. Am. Chem. Soc.* 75 (1953) 1732.
14. Dieckmann, W. and Stein, R. *Ber.* 37 (1904) 3370.
15. Forsén, S. and Nilsson, M. *Acta Chem. Scand.* 13 (1959) 1383.
16. Berliner, E. *Org. Reactions* 5 (1949) 229, 263.
17. Eistert, B., Haupter, F. and Schank, K. *Ann.* 665 (1963) 55.
18. Nef. J. U. *Ann.* 277 (1893) 59.

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On the Metabolism of Sorbic Acid in the Mouse

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[1-^{14}C]Sorbic acid has been prepared, and its metabolism in mouse has been studied. The main metabolites are carbon dioxide and water. Small amounts of muconic acid and sorbic acid were found in the urine.

Sorbic acid, $CH_3CH{=}CHCH{=}CHCOOH$, is an antimicrobial agent, which is used in increasing quantities as a food preservative. This use has led to a marked interest in the metabolism of the compound. Deuel *et al.*[1] in 1953 published an investigation on the fate of sorbic acid in rat, which showed that the intermediary metabolism was similar for sorbic, caproic, and butyric acids. From these results the authors concluded that the end products of the oxidation of sorbic acid in rat are carbon dioxide and water only. In 1962, when the present investigation on the metabolites of [1-^{14}C] sorbic acid in mice was almost completed, Fingerhut, Schmidt and Lang[2] published an extensive examination of the metabolism of [1-^{14}C]sorbic acid in rats. They found that during 4—10 h 85 % of the radioactivity were exhaled as $^{14}CO_2$ and 1.4 % excreated in the urine as $NH_2{}^{14}CONH_2$, 0.2 % as $^{14}CO_2$. No active sorbic acid or muconic acid were found in the urine. The present inves-

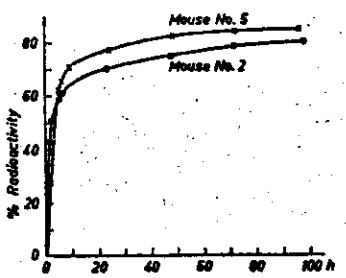

Fig. 1. Radioactive carbon dioxide expired after the administration of [1-^{14}C] sorbic acid to mouse.

Table 1.

Mouse No.	Sorbic acid given		Radioactivity in % of the given activity found during 4 days in							Time for half the activity given to be expired, hours	Muconic acid activity in % of administered activity	Sorbic acid in the urine in % of given sorbic acid
	mg/kg body weight	counts/min per mouse	the carbon dioxide expired	urine					feces			
				1st day	2nd day	3rd day	4th day	total				
1	40	640 000	82	2.3	0.2	0.1	0.04	2.6	0.9	2	0.2	
2	40	640 000	81	2.6	1.0	0.6	0.1	4.3	0.9	2	0.3	0.7
3	40	640 000	77	2.7	0.4	0.25	0.1	3.5	1.0	5		
4	40	640 000	78	2.7	0.9	0.3	0.2	4.1	0.6	2.5		
5	3000	850 000	85	4.5	0.4	0.1	0.1	5.1		3.5	0.6	0.7
6	3000	850 000	80	4.8	0.5	0.05	0.05	5.4		8	0.5	

tigation differs from that of Fingerhut *et al.* in experiment animals, experimental conditions and also in results.

Mice were given water solutions of the sodium salt of [1-^{14}C]sorbic acid by gastric intubation. Contrary to the animals of Fingerhut *et al.* they got water and food *ad libitum*. The expired, radioactive carbon dioxide was collected in sodium hydroxide solution during 4 days. It was precipitated as barium carbonate, the activity of which was measured in "infinitely" thick layers. The amounts of radioactive carbon dioxide expired are shown in Fig. 1 and Table 1. There was still a slow expiration of radioactive carbon dioxide at the time the measurements were interrupted.

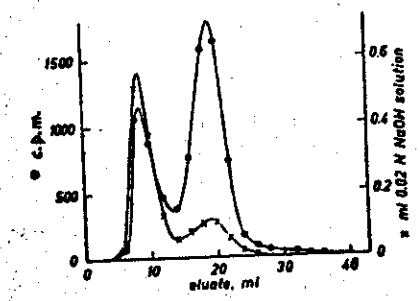

Fig. 2. Partition chromatography of the urinary metabolites of [1-^{14}C]sorbic acid.

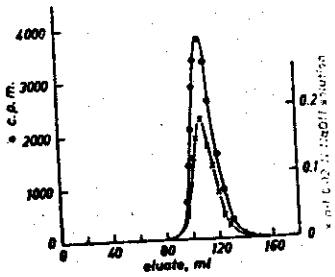

Fig. 3. Partition chromatography of [1-^{14}C] sorbic acid.

Table 2.

Recrystallization	Specific activity, c.p.m./mg
1	146
2	124
3	120
4	133
5	134
6	129

For each animal the total radioactivity of the neutralized urine was measured daily on thin layers of evaporated aliquots. About 4 % of the radioactivity administered to the mice were found in the urine during 4 days. Part of the metabolites (30–35 % of the radioactivity in the urine sample) could be extracted with ether from the acidified urine of the first 24 h and separated by partition chromatography. The activity in the ether solution was distributed amongst at least two different metabolites (Fig. 2). The compound or compounds giving rise to the first activity peak are probably conjugates, but they have not been identified. The main ether extractable metabolite (second peak, 0.2–0.6 % of the total activity given to the mouse) has been identified as *trans-trans*-muconic acid, HOOCCH=CHCH=CHCOOH, both by chromatography with addition of unlabelled muconic acid, when the activity peak and the alkali titration peak were in the same fractions (Fig. 4), and by repeated crystallization from water and ethanol with inactive muconic acid, when the specific activity soon remained constant (Table 2).

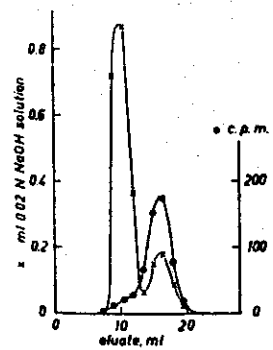

Fig. 4. Partition chromatography of the substance from the second peak of Fig. 2 together with a small amount of unlabelled *trans-trans*-muconic acid and a great amount of unlabelled malonic acid.

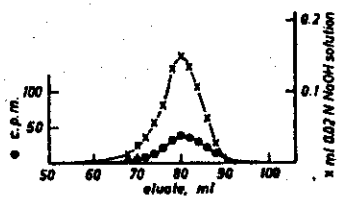

Fig. 5. Partition chromatography of part of an extract of a first day urine to which unlabelled sorbic acid has been added.

A small amount of unchanged sorbic acid was also found in the ether extract of the urine from the first 24 h. In the chromatogram (Fig. 2) it has been lost on account of its volatility and oxidability. The acid could be retained by inactive sorbic acid added to the urine before the ether extraction. When the evaporated ether extract was then chromatographed, the titration peak of sorbic acid was found in the same fractions as a radioactivity peak (Fig. 5). Quantitative microanalysis of sorbic acid on a steam distillate of the urine from mice given sorbic acid gave the amounts of sorbic acid present (about 0.7 % in the urine from the first 24 h).

The activity of the feces was measured daily for each animal. Thin layers of evaporated ether extracts, obtained after alkaline digestion and acidification of the feces, were used for the measurements. The fecal, ether extractable radioactivity being only about 1 % of the activity given to the mice, no efforts have been made to identify the active products.

Thus in 4 days 81 % ± 10 % of the sorbic acid given was oxidized to carbon dioxide and water. About 4 % of the activity left the mice with the urine, part of it as muconic acid (in the urine from the first 24 h about 0.4 %) another part as sorbic acid (in the urine from the first 24 h about 0.7 %).

EXPERIMENTAL

Preparation of [1-^{14}C]sorbic acid. A sorbic acid labelled with ^{14}C in the 1 position was prepared by refluxing 0.102 g of [1-^{14}C]malonic acid, 0.080 ml of crotonic aldehyde, and 0.105 ml of pyridine for 2½ h (*cf.* Miller and Nord [3]). The solvent was evaporated under reduced pressure, and the residue was purified by chromatography on equal parts of kieselguhr (Hyflo supercel) and silica gel with benzene as solvent. In order to diminish the loss of radioactive acid in the column, 0.025 g of pure, inactive sorbic acid was placed on the column together with the reaction product.

The [1-^{14}C]sorbic acid obtained was further purified by repeated crystallization from 50 % ethanol and by chromatography on hydrophobic kieselguhr (Hyflo supercel treated with gaseous dichlorodimethylsilane, washed with 10 % ethanolic acetic acid and ethanol and dried at 110°–120°). The stationary phase was butanol and the moving phase water.

A portion of weakly labelled [1-^{14}C]sorbic acid has been bought. The absence of radioactive impurities is seen from Fig. 3, which shows the radioactivity and titration peaks when the product was chromatographed as described above.

Stock solutions and administration of [1-^{14}C]sorbic acid to mouse. Stock solutions of sodium [1-^{14}C]sorbate (0.136 % and 15.5 %) in water were prepared from the two labelled sorbic acid samples. For radioactivity determinations the solutions were diluted with ethanol, aliquots were plated on copper planchets and measured with a gas flow counter (background 45 c.p.m.) in "infinitely" thin layers. The activities of the solutions were found to be 1 280 000 c.p.m./ml and 1 700 000 c.p.m./ml.

The animals used in this investigation were white, adult, female mice, weighing about 22 g. 0.5 ml of a stock solution (640 000 c.p.m. or 850 000 c.p.m.) was given by gastric intubation to each mouse (unanesthetized). Before and during the experiments the usual food and water were fed *ad libitum*.

^{14}C recovery in carbon dioxide expired after administration of [1-^{14}C]sorbic acid. Each mouse given a ^{14}C-sorbate stock solution was immediately placed in a metabolism cage for mice according to Roth *et al.* [4] Through the cage a stream of CO_2-free air was passed at a constant rate of about 225 ml/min. The air with the radioactive carbon dioxide expired by the mouse was passed through two effective absorption vessels containing 3 N sodium hydroxide solution (50 ml in each) for collection during 1 h or 50 % potassium hydroxide solution (50 ml in each) for collection during 24 h. The carbonate formed was precipitated from the boiling solution with 2 N barium chloride solution in the presence of ammonium chloride. The activity of the barium carbonate was measured with

an automatic flow counter in "infinitely" thick layers. The results are seen in Fig. 1 and Table 1.

Isotope recovery in the urine. Identification of the main isotopic compounds extractable with ether from urine collected during the first 24 h after the administration of [1-^{14}C] sorbic acid. For each mouse the urine was collected daily during 4 days after the administration of the labelled sorbic acid. The urine samples were diluted to 5 ml each. From each sample 0.2 ml was neutralized and evaporated on a copper planchet, and the activity was measured. The results are seen in Table 1.

The urines obtained during the first 24 h after the administration of the [1-^{14}C] sorbic acid were acidified (pH < 2) and extracted 3 times with equal volumes of ether in order to find out if other active products than carbonate and urea, already pointed out by Fingerhut *et al.* in rats, were present in the urine. The combined ether extracts were dried with anhydrous sodium sulphate and evaporated at reduced pressure just to dryness. Aliquots of the residue were chromatographed on hydrophobic kieselguhr (3.5 g) with butanol (3.0 ml) as stationary phase and water as moving phase. Fractions of 1.5 or 2 ml were collected, titrated (Agla micrometer syringe) with 0.02 N sodium hydroxide solution, using thymol blue as indicator, and then evaporated to dryness at 85°–105°. The residues in the tubes were dissolved in 50 % ethanol, evaporated on copper planchets, and the radioactivities were counted. The active peaks obtained are seen in Fig. 2.

Part of the second peak of the chromatogram of Fig. 2 was rechromatographed together with a small amount of unlabelled *trans-trans*-muconic acid and a great amount of unlabelled malonic acid on hydrophobic kieselguhr (4.5 g) with chloroform–2-ethylhexanol–methanol (4 ml) as stationary phase and water–methanol as moving phase. Water (180 ml), methanol (170 ml), chloroform (25 ml), and 2-ethylhexanol (25 ml) were equilibrated to form the phases. Fractions were collected and treated as in the preceding chromatogram. The radioactivity peak and the acidity peak, which emanated from the muconic acid, were found in the same fractions (Fig. 4).

The definite proof that the second peak really contained radioactive *trans-trans*-muconic acid was obtained by repeated crystallization of the fraction concerned with inactive *trans-trans*-muconic acid alternately from water and ethanol and determination of the specific activity of the crystals. Table 2 shows that the specific activity soon became constant, which demonstrates the identity of the labelled and unlabelled products.

No sorbic acid was found in the chromatograms of the ether extracts of the urine of the first day (no radioactive peak at 90–130 ml of eluate; *cf.* Fig. 3), but sorbic acid being rather volatile and also easily oxidized might be present in spite of that. A special search for sorbic acid has therefore been made.

To a 1st day urine were added 10.4 mg of unlabelled sorbic acid, and the urine was extracted 3 times with ether. The ether extract was evaporated just to dryness under reduced pressure, and part of the residue was chromatographed on hydrophobic kieselguhr (3 g) with butanol (2 ml) as stationary phase and water as moving phase. The fractions were titrated and the radioactivity of their dry substance was measured. As seen from Fig. 5 the titration peak of the unlabelled sorbic acid is in the same fractions as a radioactivity peak.

A quantitative analysis of the sorbic acid percentage in the urine was also performed according to Schmidt.[5] 0.7 % of the sorbic acid administered to the mice were found in the urine from the first 24 h.

Isotope recovery in feces after administration of [1-^{14}C]sorbic acid. The feces from each mouse were collected daily during 4 days after the administration of the labelled sorbic acid. They were homogenized and hydrolysed with boiling 5 N ethanolic potassium hydroxide solution for 30 h. The alcohol was evaporated, the residues were acidified with 6 N hydrochloric acid and extracted three times with ether. The ether solutions were evaporated to dryness and the residues were dissolved in 50 % ethanol. The activities were measured on evaporated aliquots. In the feces from the first two days there was found about 1 % of the radioactivity given (mean of 5 mice). In the feces from the third and fourth days the activities were negligible.

I am indebted to Mrs. M. L. Errind and Mrs. D. Sjögård for valuable technical assistance.

REFERENCES

1. Deuel, H. J., Jr., Calbert, C. E., Anisfeld, L., McKeehan, H. and Blunden, H. D. *Food Res.* 19 (1954) 13.
2. Fingerhut, M., Schmidt, B. and Lang, K. *Biochem. Z.* 336 (1962) 118.
3. Miller, R. E. and Nord, F. F. *J. Org. Chem.* 16 (1951) 1720.
4. Roth, L. J., Leifer, E., Hogness, J. R. and Langham, W. H. *J. Biol. Chem.* 176 (1948) 249.
5. Schmidt, H. *Z. anal. Chem.* 178 (1960) 173.

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Constituents of Umbelliferous Plants

IV *. The Coumarins of *Peucedanum palustre* L. The Structure of a New Coumarin

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A coumarin, $C_{19}H_{20}O_5$, obtained from the root of *Peucedanum palustre* is shown to be (+)-8-(1-angeloyloxy-1-methylethyl)-8,9-dihydro-2H-furo[2,3-*h*]-1-benzopyran-2-one (I). (I) is shown to possess the same configuration at C-8 as athamantin (II) and archangelicin (III).

Furthermore, the root afforded isoimperatorin (IV), (+)-oxypeucedanin (V), ostruthol (VI), and (+)-oxypeucedanin hydrate (VII).

Peucedanum palustre is widely distributed in the Eastern and Middle Europe, but has so far not been investigated chemically. This paper presents the results from an investigation of the coumarin content.

Besides isoimperatorin IV, (+)-oxypeucedanin (V), ostruhol (VI), and (+)-oxypeucedanin hydrate (VII) a blue-fluorescent compound (I), $C_{19}H_{20}O_5$, m.p. 118.5—119°, was isolated.

Its coumarin character was strongly indicated by the fluorescence, the absorption bands in the infrared at 1719—1736, 1624, 1584, 1495, and 1459 cm^{-1}, and by its UV-absorption: λ_{max} 218 mμ (4.31) (shoulder), 251 mμ (3.41) 261 mμ (3.46), and 328 mμ (4.20). λ_{min} 245 mμ (3.35), 255 mμ (3.36), and 268 mμ (3.12). The UV-spectrum is similar to that of archangelicin (III) formerly investigated by the present authors.[1]

Treatment of (I) with 85 % phosphoric acid, afforded angelic acid, dihydrooroselone (VIII), and an optical active coumarin (IX) with the empirical formula $C_{14}H_{14}O_4$. The UV-spectrum of (IX) was very similar to that of (I). Saponification of (I) with 1 N sodium hydroxide also afforded (IX). On catalytic hydrogenation of (I) 2 moles of hydrogen were consumed. Subsequent saponification afforded (+)-tetrahydrooroselol (X), a compound earlier prepared on hydrogenation of athamantin (II) by Halpern *et al.*[2] This evidence clearly identifies compound (IX) as (+)-8,9-dihydrooroselol.

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On the biological oxidation of polyunsaturated fats

A new method for the preparation of polyene-dicarboxylic acids (1)

By Richard Kuhn, Frenz Koehler and Leonore Koehler

Since it has become possible to synthetize (2) polyunsaturated fats, the aliphatic polyene-carboxylic acids (I)

I $H_3C - (CH-CH)_n - COOH$

we have made tests about their behavior in the animal body. We were interested particularly in a comparison with the saturated fatty acids which have also been frequently the subject of studies in the form of their esters and glycerides. It was to be expected that the polyene-carboxylic acids, corresponding to the fatty acids according to Verkade, would pass over with a suitable chain length into polyene-omega, omega'-dicarboxylic acids (II)

II $HOOC-(CH=CH)_n - COOH$

of which no higher member than muconic acid (n = 2) was known at the start of our investigation.

After feeding sorbic acid (I, n = 2) to rabbtis, we could indeed isolate from the urine trans-trans-muconic acid, which was converted into the dimethyl ester with a melting point of 157 degC 9mixed sample). The yield (0.1 - 0.2%) was very low, however, From sorbic methyl ester we obtained no dicarboxylic acid at all, from sorbic ethyl ester we obtained 0.5%. The picture changed markedly when we switched to nitrogen-containing derivatives. From the urine of rabbits which had been fed sorbic amide we isolated in a yield of 32% of the theory a compound $C_6H_7O_3N$ with amelting point of 281-282 degC which crystallized in shining, white needles. This compound proved to be the still unknown muconamic acid $HOOC-CH=CH-CH-CH-CO.NH_2$. It could be easily saponified to trans-trans-muconic acid, melting point 299 degC (mixed sample) with alcoholic potash lye, giving off ammonia. Even more productive was the omega-oxidation of sorbic methyl-amide from which we obtained44% of the theory of pure mucomethylamic acid $HOOC-CH=CH-CH-CH-CO.NH.CH_3$ with a melting point of 217 degC. From sorbic anilide we obtained up

to 36% of the theory of muconanilic acid HOOC-CH=CH-CH=CH-CO.NH.C6H5, melting at 263 degC.

The oxidation of the end-standing methyl group is extremely enhanced by converting the polyene-carboxylic acid into its amide,methylamide, anilide etc. The yields are so good that the rabbit will be used for the preparation of some otherwise hardly obtainable compounds. From beta-methyl sorbic amide we obtained, for example, in a yield of 62% of the theory the beta-methyl-muconamic acid HOOC-CH=CH-C(CH3)=CH-CO.NH2 in long prisms with a melting point of 259-261 degC. If we wanted to produce this substance from the beta-methyl-muconic acid, it would not be easy to bring the amide group only to the carboxyl adjoining the methyl group,

Determinant for the high yield seem to us

1. the elimination of the beta-oxidation by the formation of the amide of the carboxyl group
2. the reduction of the combustion rate by the conjugated double bonds which can already be recognized without amidation
3. the further inhibition of the beta-oxidation which produces the beta-standing methyl group (beta-methyl-sorbic acid) found in some cases (3).

The influence of the amidation is surprising. B. Flaschentraeger (4) found in feeding dogs that 61% of the sebacic acid HOOC-(CH2)8-COOH were excreted unchanged from the urine, while sebamic acid HOOC-(CH2)8-CONH2 was only excreted at the rate of 10%. The amide had thus been easier decomposed than the acid. In our cases the opposite is true.

After feeding crotonic anilide $C_{10}H_{11}ON$, neither maleic- or fumaric anilic acid could be isolated from the urine. We obtained rather a compound $C_{10}H_{11}O_2N$ in a yield of 14% of the theory which melted at 189-190 degC and which therefore contains 1 oxygen atom more than the starting material. In the mild catalytic hydration 1.06 moles H2 were absorbed. The dihydro substance $C_{10}H_{13}O_2N$ melted at 138 - 139 degC. To compound $C_{10}H_{11}O_2N$ showed

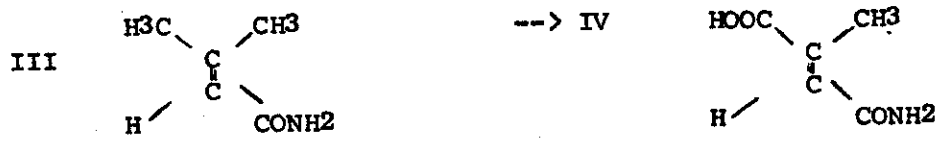
green coloration in methanol with ferric chloride, was soluble in soda lye and manifested itself thus as a phenol. Since acetanilide and other derivatives of aniline are oxidized in the animal body to derivatives of p-aminophenol (5) it could be assumed that the substance isolated from the urine is N-crotonyl-p-aminophenol·H3C-CH=CH-CO-NH-C6H4-OH. The synthesis of this substance from p-aminophenol and crotonic chloride proved the accuracy of the assumption (mixed sample). The dihydro compound $C_{10}H_{13}O_2N$ is identical in all properties with the N-butyryl-p-amino-phenol H3C-CH2-CH2-CO-NH-C6H4-OH obtained by us from butyric chloride and p-aminophenol. This is the only case of excretion of a p-amino-phenol derivative which we encountered in the course of the present investigation in feeding tests with anilides. As a starting member of the homologous series of the polyene dicarboxylic acids, crotonic acid also holds biologically an exceptional position.

The amide of beta-beta-dimethyl acrylic acid $(H3C)_2C$=CH-COHN2 yielded a carboxylic acid C5H7O3N, with a melting point of 223 degC, in a yield of 43% of the theory, which proved to be identical with the mesaconic-alpha-amide acid (melting point 222 degC) described by R. Anschuetz (6). This finding is of stereochemical interest, since not only mesaconic acid (trans-form) but also citraconic acid (cis-form) can be formed by methyl oxidation of beta-beta-dimethyl-acrylic acid. In the oxidation in the animal body, apparently only the methyl group is attacked which is end-standing relative to the carbon amide group.

In this connection it should be pointed out that in comphor, too, only that methyl of the two methyls is attacked at the bridge during the formation of pi-oxycomphor (Y.Ashina and M. Ishidate (7)) which is trans-standing to the carbonyl group. If more general principles should apply here, it would be possible to obtain new clues for the correlation of cis-trans formulas. Just as from dimethyl-acrylic acid, two different cis-trans-isomeric Hildebrandt acids can also be formed from geranic acid, depending on which of the two methyls is oxidized in the end-standing isopropylidene radical by the animal

body to the carboxyl. Which of these is present in the compound with a melting point of 194 degC isolated from the urine could not be determined in our last study (8). The results obtained so far make it likely that we are dealing here with the transform.

The constitution of the mesaconic-alpha-amide acid was disclosed by R. Anschuetz (9) after long and laborious investigations. The main problem was the decision which of the two ester acids HOOC-C(CH3)=CH-COOCH3(alpha) and H3COOC-C(CH3)=CH-COOH(beta) melts at 52 and 84 degC resp. The conclusion that the methyl group adjoins the free carboxyl group in the lower melting alpha form resulted from a synthesis based on gamma-dibromo-alpha-methyl-acetacetic ester. The accuracy of the formulas assigned by R. Anschuetz to the alpha- and beta-compounds is verified in the rabbit in a very simple manner: the mesaconic-alpha-amide acid obtained from dimethyl-acrylic amide can carry the methyl group only adjacent to the free carboxyl group.

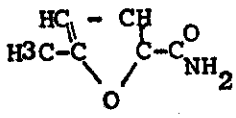
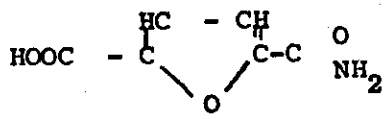


Surprisingly no carboxylic acids which are capable of passing into the urine could be obtained from beta-methyl-beta-ethyl-acrylic acid

V (H3C) (H5C2)C = CH=COOH

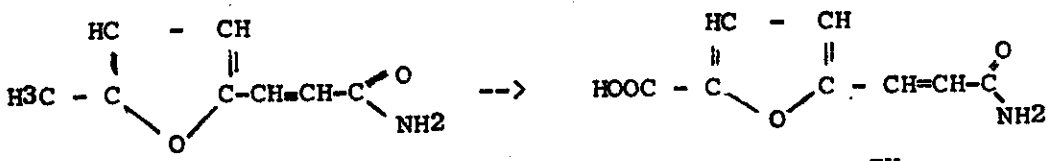
which was fed both as ethyl ester and as amide (melting point 128 - 128.5 degC).

The more favorable were the tests with polyene-carboxylic amides where two conjugated double-bonds are combined by an oxygen bridge to a furane ring. From a study by K.W. Hausser, R. Kuhn, A. Smakula and A.Deutsch (10) it was known that the furane ring is equivalent in optical respect (position of the absorption bands) to an open system of two conjugated double bonds. We checked whether this equivalence also applies biologically to the oxidation processes in the animal body. From 5-methyl-pyromucic amide (VI) we obtained in a yield of 32% of the theory the furane-dicarboxylic-(2,5)-amide acid (VII)with a melting point of 284 degC.

VI  VII 

We owe to B. Flaschentraeger (11) the discovery that small amounts of furane-dicarboxylic acid-(2,5) namely 3-5 mg per day, apparently form a normal constituent of the human urine. The amide (VII) isolated by us was converted by saponification with alcoholic potash lye into furane-alpha, alpha'-dicarboxylic acid whose dimethyl ester showed the correct melting point of 109 degC.

The beta-(5-methyl-furyl-(2)-acrylic amide (VIII) showed a record yield of 83% of the theory. Furane-acrylic amide-(2)-carboxylic acid-(5) (IX) with a melting point of 280 degC



VIII IX

The acrylic analogon, the octatrienic acid (X)

X H3C - CH=CH - CH=CH - CH=CH - COOH

fed as sodium salt yielded no dicarboxylic acid. But its amide (XI) led to the excretion of a difficultly soluble carboxylic acid C8H903N which melted at 263 degC under decomposition, of which 42% of the theory were obtained. By saponification with aqueous potash lye we obtained therefrom the hexatriene-1,6-dicarboxylic acid (XIII). The compound isolated from the urine represents thus the hexatriene-2,4,6-decarboxylic-1,6-amide acid-1 (XII).

Xi H3C-CH=CH-CH=CH-CH=CH-CONH2

XII HOOC-CH=CH-CH=CH=CH=CH-COHN2

XIII HOOC-CH=CH-CH=CH-CH=CH-COOH

The hexatriene-1,6-dicarboxylic acid (XIII) was unknown until recently. It was synthetized for the first time a few months ago from oxalosorbic ester (12). For positive identification we converted the dicarboxylic acid obtained from the urine with phosphorus pentachloride and methanol into the dimethyl ester, which melted at 171 degC and which showed no reduction of the melting

point with the hexatriene-1,6-dicarboxylic-dimethyl ester by R.Kuhn and Ch. Grundmann (melting point 171.5 degC).

In the decatraenic acid (XIV) the sodium salt

XIV $H_3C-CH=CH-CH=CH-CH=CH-CH=CH-COOH$

is already so difficultly soluble that we used the practically completely insoluble free acids for feeding. Neither the starting substance nor a decarboxylic acid could be obtained from the urine of the rabbit. But when we changed to the amide XV), we could obtain a very difficultly soluble carboxylic acid in a yield of 20% of the theory by acidifying the urine. This is octatetraenic-2,4,6,8-dicarboxylic-1,8-amide acid (XVI), because it could be saponified by aqueous potash lye to the yellow octatetraenic-1,8-dicarboxylic acid (XVII).

XV $H_3C-CH=CH-CH=CH-CH=CH-CH-CH-CONH_2$

SVI $HOOC-CH=CH-CH=CH-CH=CH-CH=CH-CONH_2$

XVII $HOOC-CH=CH-CH=CH-CH=CH-CH=CH-COOH$

The proof of the constitution is based in this case too on the comparison of the dimethyl esters. The octatetraenic-1,8-dicarboxylic-dimethyl ester obtained from the urine melted at 212 degC, the preparation obtained according to the oxalo-ester-synthesis by R. Kuhn and Ch. Grundmann (12)melted at 213 degC. The eutectic melting point was 213 degC.

If we compare the yields of omega-dicarboxylic acids, which can be obtained, on the one hand, from fatty acids and, on the other hand, from polyene-carboxylic acids, regarding the dependence on the number of C-atoms in the chain, we find: From the fatty acids we obtain the maximum yield of dicarboxylic acid with 10 C-atoms (man, (13)) and with 8 C-atoms resp. (dog(14)) from the amides of the polyene-carboxylic acids with 8 C-atoms (rabbit).

4 C-atoms:	crotonic amide	an. 0% of the theory
6 C-atoms:	sorbic amide	32%
8 C-atoms	octatrienic amide	42%
10 C-atoms	octatetraenic amide	20%

In the drop from trienic to tetraenic acid we have to keep in mind that the unfavorable solubility conditions, and thus probably also the unfavorable resorption ratios increase with increasing number of conjugated double bonds. The great drop from sorbic acid to crotonic acid may be partly due to the considerably toxicity of the crotonic amide, which had led to the loss of many rabbits, though only relatively small amounts were fed. We want to point out that some other amides, methyl-amides and anilides showed partly great pharmaceutical effects on the rabbits, which will be discussed more fully in the experimental part. Paralytic symptoms, but also spasms, mostly starting at the rear legs, were prominent. In some case there was complete narcosis.

Description of the tests

I. Preparations

1. Hexadiene-(2,4)-acid-(1); sorbic acid (I, n = 2) From crotonic aldehyde according to O.Doebner (15). Yield of acid with defined melting point: 25% of theory. Melting point 185 degC (corr. theory).

2. Hexadiene-(2,4)-acid-(1)-methyl ester According to O. Doebner and A. Wolff (16). Yield 99% of theory. Boiling point 20 = 71degC

3. Hexadiene-(2,4)-acid-(1)-ethyl ester (16). Yield 81% of theory. bp-11 = 81.5 degC, $n_D^{19} = 1,4942$.

4. Hexadiene-(2,4)-acid-(1)-amide (16). Yield 91% of theory,melting point 171.5 - 172.5 degC (corr.theory).

5. Hexadiene-(2,4)-acid-(1)-methyl amide. 52 g fresh vacuum-distilled sorbic chloride were diluted with dry ether, cooled with acetone-dry ice and mixed slowly with a small excess of liquid methylamine. After evaporation of the ether, the residue was boiled thoroughly with acetone. The sorbic methylamide separated by the evaporation of the acetone was recrystallized from diluted alcohol and large colorless platelets were obtained. Yield 50 g (100% of the theory), melting point 141 degC (corr. theory, sublimated). The substance is soluble in acetone, alcohol, methanol and hot water.

4.167 mg substance: 10.30 mg CO2, 3.40 mg H20 - 3.688 mg substance: 10.49 cc N2 (21 degC, 754 mm).

$C_7H_{11}ON$ (125.1)	calc.	C 67.14	H 8.87	N 11.19
	found	67.41	9.13	1049

6. Hexadiene-(2,4)-acid-(1)-anilide. According to O.Doebner and A.Wolff(17). Yield 72% of the theory, melting point 155-156 degC (corr.theory).

7. Methyl-(2)-hexadiene-2,4)-acid-(1)-amide; beta-methyl-sorbic amide.

a) Pentanol-4-on-(2). We obtained this substance according to the data of a patent by I.G. Farbenindustrie (French patent 626,729 (17)), but added prior to the distillation some hydroquinone so that the yields were improved. 300 g dry acetone were mixed with 90 g acetaldehyde. Under intensive cooling with an ice-common salt mixture, we added gradually to the cooled reaction mixture 6 g finely powdered caustic potash with some hydroquinone, and the mixture was left standing over night in the refrigerator under exclusion of water. Then it was treated with carbon dioxide, filtered off from the separated bicarbonate, and fractioned. Bp-14 60-70 degC.

Rough yield 50 g (24 % of the theory related to acetaladehyde).

b) Pentene-(2)-on-(4); Ethyledene-acetone. The dehydration of the epntanolon was effected with 1½ moles acetic anhydride and a trace of zinc chloride. We heated for 60 minutes in the oil bath to 150-160 degC and obtained after preparation in known manner the ethyledene-acetone in a yield of 49-52% of the theory.

c) Methyl-(2) hexadienic-(2,4)-acid-(1). The beta-methyl-beta-oxy-DELTA gamma, delta-hexenic-methylester obtained according to R. Kuhn and M. Hoffer (18) was heated for 20 minutes to about 80 degC with anhydrous sodium bisulfate (1/3 of the weight of the oxyester) and subjected subsequently to vacuum distillation. Yield 50% of the theory. The saponification of the ester was effected with 1½ moles 10% alcoholic potash lye.

d) Methyl-(2)-hexadienic-(2,4)-amide. The acid was treated in known manner with thionyl chloride, mixed after cooling in acetone-dry ice with liquid

ammonia; then the separated amide was treated with hot acetone and filtered off from the undissolved ammonium chloride. The filtrate was boiled with animal charcoal, filtered, and left standing for crystallization. Melting point 136-141 degC.

8. Octatrienic-(2,4,6)-acid-(1). (I, n = 3). From hexadienal according to R. Kuhn and M. Hoffer (19), purified with ammonia. The hexadienal was prepared according to themeth od indicated by R. Kuhn, W. Badstuebner, and Ch. Grundmann (20). The acid recrystallized once from diluted methanol melted at 184.5 degC (corr. theory).

9. Decatetraenic-(2,4,6,8)-acid-(1). (K, n = 4). According to R. Kuhn and W. Hoffer (21). The octatrienal was obtained by using piperidine-glacial acetic acid as a catalyst. The acid recrystallized once from glacial acetic acid melted at 222 degC (corr. theory).

10. Octatrienic-(2,4,6)-acid-(1)-amide (X,I). 30 g trienic acid were mixed with 28 g freshly distilled thionyl chloride and heated for 30 minutes on the steambath under reflux (calcium chloride tube). Then the excess thionyl chloride was eva;orated in the vacuum, 150 cc dry ether were added, the product was cooled thoroughly in an acetone-dry ice mixture, and liquid ammonia was carefully added to the ether solution. After evaporation of the ether and of the excess ammonia, the residue was extracted with water, the acid amide was filtered off and recrystallized from boiling alcohol. Yield 20.2 g; from the mother liquor we obtained an additional 5.9 g. The substance crystallizes from hot water in white, feathered, flat needles which melt at 208-209 degC without decomposition.

4.020, 3.122 mg substance: 10.34, 10.60 mg CO2, 2.95, 3.02 mg H20.

4.261, 4.227 mg substance: 0.381 (21deg, 741 mm) 0.383 (21deg, 743 mm)ccN.

$C_8H_{11}On$ (137.1)	calc.	C 70.02	H 8.09	N 10.21
	found	70.15;70.13	8.21;8.19	10.12; 10.28

11. Decatetraenic-(2,4,6,8)-acid-(1)-amide (XV). 15 g tetraenic acid with a melting point of 224 degC (corr.theory) were finely ground and, after the addition of 40 cc benzene (dried over sodium) mixed with 16 g freshly distilled thionyl chloride. After heating for 35 minutes on the steambath, the mixture was liberated of the excess thionyl chloride by means of a vacuum pump and filtered off from the unreacted tetraenic acid (2.5 g). The filterate was cooled to -15 degC and mixed with excess liquid ammonia and after the reaction it was filtered off immediately from the precipitate. The latter was treated with cold water to remove the ammonium chloride, and the residue was recrystallized with cold water from absolute alcohol. Yield 9.6 g. The substance crystallized from alcohol in orange-colored prisms which melt at 227 degC (corr. theory) without decomposition.

4.174 g substance: 11.18 mg CO2, 2.99 mg H20 . 3.584 mg substance: 0.282 cc N (20 deg., 750 mm).

$C_{10}H_{13}ON$ (163.1)	calc.	C 73.57	H 8.03	N 8.57
	found	73.38	8.05	8.41

12. Crotonic amide Deviation from R. Stoermer and H. Stockmann (22) we obtained the crotonic chloride not over the sodium salt, but from the free acid with thionyl-chloride. Yield of the acid amide 93% of the theory. From acetone the substance crystallizes in long needles, from water in square platelets. Melting point 161.5 degC (corr. theory).

13. Crotonic anilide. The acid chloride obtained from the free acid with theionyl chloride, which boils at 121-123 degC under normal pressure, was reacted with ice-cooled, freshly distilled aniline. The anilide crystallizing from moderately diluted alcohol in beautiful needles melted at 117-118 degC (corr.theory). Yield of substance with a defined melting point 80% of the theory.

14. Beta, beta-dimethyl-acrylic amide (III). The acid chloride was prepared according to H. Staudinger and E. Ott (23). Yield 98% of the theory. Bp-760 = 145-148 degC. To the acid chloride diluted with dry ether we added

slowly in drops liquid ammonia under cooling with acetone-dry ice. After evaporation of the excess ammonia and ether, the filtrate was heated with animal charcoal, filtered again, and left standing for crystallization. Yield (including the acid amide obtained from the mother liquors) 89% of the theory. From acetone: large plates, from water : long, colorless, coarse needles. Melting point 110-111 degC (corr.theory). The substance is insoluble in petroleum ether, easily soluble in acetone, methanol, alcohol, ether and hot water.

4.345, 4.286 mg substance 9.675, 9.560 mg CO_2, 3.575, 3.510 mg H_2O.

C_5H_9ON (99)	calc.	C 60.61	H. 9.09
	found	60.73;60.83	9.21; 9.16

15. Beta-methyl-ethyl-acrylic amide

a) Beta-methyl-beta-ethyl-acrylic amide (V). H.D. Gardner and W. Haworth (24) prepared this acid by condensation of methyl-ethyl-ketone with sodium-cyano-acetic ester and subsequent saponification and decarboxylation of the condensation product. We obtained it by condensation of methyl-ethyl ketone with bromoacetic ester and zinc according to S. Reformatsky. 72 g methyl-ethyl-ketone were mixed with 65 g zinc chips, 200 cc dry benzene, and 153 g bromoacetic methylester. During the heating on the steambath the reaction set in very vigorously after a short induction period, so that the mixture had to be cooled by shaking in cold water. After the voluntary boiling was completed, the heating was continued for ½ hour. Then the mixture was cooled and decomposed with ice and a slight excess of diluted sulfuric acid. After washing the benzene layer with water, it was dried over sodium sulfate and distilled in fractions. The beta-methyl-beta-oxy-valeric-methylester passed over under 12 mm at 74-78 degC. $n_D^{20.5} = 1.4487$. But we also observed boiling points of 102-107 degC (12 mm). Perhaps the cis-trans-isomeric forms are formed side by side.

b) Beta-methyl-beta-ethyl-acrylic amide. 38 g acid with a bp-12 of 107.5 - 109 degC were heated on the steambath for 30 minutes with 100 cc dry ether and 42 g freshly distilled thionyl chloride, and the reaction mixture turned brown. After the reaction was completed, the ther was evaporated and the residue distilled in the vacuum. The beta-methyl-beta-ethyl-acrylic chloride boils under 13 mm at 48 degC; $n_D^{21} = 1.3534$. Yield 43.8 g (99% of the theory).

The distilled acid chloride was diluted with 150 cc dry ether, cooled in acetone-dry ice, and a slight excess of liquid ammonia was slowly added. After evaporation of the solvent the residue was treated with hot acetone, filtered off from the undissolved ammonium chloride and the filtrate was concentrated and the amide crystallized. Yield: 34 g (91% of the theory). For further purification the product was recrystallized from water. The substance melts at 128 degC to 128.5 (corr. theory). It is easily soluble in hot water, acetone, alcohol, but insoluble in petroleum ether.

4.102, 4.072 mg substance: 9.575, 9.465 mg CO2, 3.565, 3.510 mg H20. 3.419 mg substance: 0.367 cc N (22 deg. 751 mm).

$C_6H_{11}ON$ (113.1)	calc.	C 63.66	H 9.81	N 12.37
	found	63.66; 63.40	9.72; 9.65	12.26

16. 5-methyl-furfurol (25) To a boiling solution of 250 g stannous chloride and 1000 g common salt in 2000 cc 17% sulfuric acid was added in drops within one hour a hot solution of 400 g cape sugar in 500 cc water. The collected distillate was made alkaline with soda and shaken out with 700 cc benzene. The benzene solution was dried over sodium sulfate. After evaporation of the solvent, the residue was distilled off in the vacuum, and 24.5 g (21% of the theory) passed over under 11 mm at 74.5 to78 degC. After redistillation, the 5-methyl-furfurol boiled under 12 mm at 75.5 degC $n_D^{19} = 1.5248$. It can be stored in the dark undecomposed for a long time under vacuum.

17. 5-methyl-pyromucic amide (VI).

a) 5-methyl-pyromucic acid. 30 g 5-methyl-furfurol were mixed with 50 cc 96% alcohol and a solution of 92.6 g silver nitrate in 100 cc water, adding in

water. After the oxidation was completed, the separated silver was filtered off, the filter was evaporated until it was almost dry, the aqueous solution was acidified and placed in the refrigerator, after it had been discolored with animal charcoal. The 5-methyl-pyromucic acid crystallizes in large rhombs and melts at 109-110 degC (corr. theory). Yield of substance with defined melting point, 79-91 % of the theory.

b) 5-methyl-pyromucic amide. 31 g 5-methyl-pyromucic acid were heated for 30 minutes on the steambath with 45 g thionyl chloride and 20 cc dry ether. After cooling, the ether solution of the acid amide was introduced slowly into the ammonia-saturated ether and the formation of the amide took place smoothly. After evaporation of the ether, the hot aqueous solution of the residue was discolored with animal charcoal and placed in the refrigerator. Yield of substance with defined melting point: 25 g (81% of the theory). Melting point 132 degC to 133 degC (corr. theory).

18. Beta-(5-methyl-furyl-(2)-acrylic amide (VIII).

a) beta-(5-methyl-furyl-(2)-acrylic acid. 28 g 5-methyl-furfurol were mixed with 27 g malonic acid and 28 g pyridine and left standing overnight at room temperature. Then the mixture was heated on the steambath under reflux until no carbon dioxide was formed anymore (about 8 hours). The reaction product was cooled with ice and acidified with diluted sulfuric acid. A brown precipitate was obtained which was dissolved in 2n-soda lye and decolorized hot with animal charcoal. The beta-(5-methyl-furyl-(2)-acrylic acid which was precipitated again with diluted sulfuric acid, was recrystallized from greatly diluted methanol, and snow-white long needles with a melting point of 157 degC (corr. theory) were obtained. Yield 28 g (70% of theory).

b) Beta-((5-methyl-furyl-(2))-acrylic amide. 40 g beta-((5-methyl-furyl-(2))-acrylic acid were heated for 90 minutes on the steambath with 39 g freshly distilled thionyl-chloride and 100 cc dry ether, and then distilled in the vacuum. Yield 44.5 g (99.5% of the theory). The beta-((5-emthyl-furyl-(2))-

acrylic chloride is a pale-yellow, lightly refractive, not unpleasantly smelling liquid which boils under 9 mm at 124 degC and which solidifies to refractive, well-defined platelets which melt at 37 degC.

The acid chloride is dissolved in ether, cooled thoroughly with acetone-dry ice, and mixed with a slight excess of liquid ammonia. The ether is evaporated and the residue treated with warm acetone, filtered off from the ammonium chloride, and the filtrate is solidified. The beta-((5-methyl-furyl-(2))-acrylic amide crystallizes from diluted acetone in prisms arranged in star-form, from absolute acetone in corase druses. Yield 38.2 g (98% of the theory). Melting point 130-131 degC (corr.theory).

Soluble in water, alcohol, methanol and acetone.

4.023, 4.187 mg substance: 9.340, 9.765 mg CO_2, 2.190, 2260 mg H_2O.

4.288, 3.995 mg substance: 0.337, 0.325 cc N (23 deg, 752 749 mm).

$C_8H_9O_2N$ (151)	calc.	C 63.57	H. 5.96	N 9.27
	found	63.32; 63.60	6.09; 6.04	8.97; 9.25

II. Feeding tests

Healthy, well-nourished rabbits were fed with the esophageal sound, and the daily dose was made depending on the type of substance.

1. Sorbic acid was dissolved in an exactly calculated amount of 2-n soda lye. The rabbit received daily twice 5 g. Pharmacological effects were not observed with this amount, and the animals survived the feeding test excellently. Weight of the animals: 3320 and 3380 g resp.

2. Octraenic- and decatetraenic acid were ground to a fine powder, and suspended in water and administered with the esophageal sound. Twice daily 1-2 g per day and animals. The animals (weight 3220 and 3320 g resp.) showed no conspicuous symptoms.

3. Sorbic methylester and -ethylester were administered diluted. Twice daily 5 g per animal. When feeding sorbic methylester, the extremely great hunger of the animals was conspicuous. (weight 3320 g) as well as the large amount of urine obtained during this test.

4. Sorbic amide The finely powdered amide was suspended in water and administered in increasing amounts of 1-4 g per meal. The pharmacological effect appearing at 4 g manifested itself in a paralysis of the legs, starting with the rear extremeities, unconscieusness and increased activity of the heart. When amounts of 3 g were fed from the beginning, an extreme delay of respiration and exitus appeared. With gradual habitation, the animals survive the test without any harm, even if temporary paralysis appears from time to time. Weight of the animal: 3320 g.

5. Sorbic anilide was finely ground, suspended in water and administered in daily doses of 2-6 g per animal. No special effect of anilide or the organism could be observed, even in feeding tests over long periods of time.

6. Sorbic methyl amide. The substance was powdered, made into a paste with water, and administered in increasing amounts of 0.5 - 2.0 g. As usual, the animals were fed twice a day. With amounts of 2.5 - 3.0 g per meal the rear legs were paralyzed after about 5 minutes, the heart beat was intensive, the breathing frequency very rapid and the breathing volume small. After 25 minutes, total paralysis and unconsciousness had set in, the heart beat very quietly, and the breathing was the same. End of the effect after about 2 hours.

7. Beta-methyl-sorbic amide. The preparation, which was not quite pure (melting point 136-141 degC) was suspended in water and administered in two doses of 0.5 and 2.0 g (within 17 hours); after a short time paralysis set in in the rear legs and spread with vigrous action over the entire body. Heart beat irregular, breathing extremely slow,breathing volume very small. Incipient end of paralysis and unconsciousness after 2 hours.

8. Crotonic amide. The high toxicity of this substance did not permit the feeding of more than 0.5 - 1.0 g per day. Nevertheless exitus occurred after feeding a total amount of 7.5 g. Weight of the animal: 3180 g. After feeding 1.5 g twice daily, exitus occurred after about 12 hours. (weight of animal: 3430g). In another test the rabbit died after 2hours already, with feedings

of 2.0 3.0 g within 7 hours (weight of animal 3320 g). Here too, paralysis was observed which lead to the characteristic spastic stretched position of the legs and of the head. In feeding tests over a longer period of tiem, necrotic changes of the kidneys are observed.

9. Crotonic anilide. The anilide was finely powdered, suspended in water, and fed in amount of twice 2 g per day. A male animal tolerated the feed without any special symptoms, while a female rabbit showed severe paralysis of the legs after the first dose of 2 g, from which it recovered rapidly and completely when the feeding test was stopped.

10. Beta-beta-dimethyl-acrylic amide (III) was finely powdered, suspended in water, and fed in increasing amounts of twice 0.5 - 2.0 g per day. The test was well tolerated by the animal without any special symptoms. Weight of the animal: 4200 g.

11. Beta-methyl-beta-ethyl-acrylic amide was fed as usual in solid form, twice daily 2 g per animal. Each time paralysis of the rear legs was observed, which remained without any further consequences, however. Weight of the animal: 2930 g.

12. 5-methyl-pyromucic amide (VI). The substance was suspended in water and fed in doses of 1 g to 2 g twice daily per animal. No pharmacological effect was observed. Weight of the animal: 3320 g.

13. Beta-((5-methyl furyl-(2))-acrylic amide(VIII) The amide was suspended in water and was fed first in amounts of 0.5 g twice a day, then we added 1.0 g and observed paralysis of the legs after 10 minutes, starting from the rear, which disappeared again after about 1 hour. With a slow increase of the dose, paralysis became increasingly weaker until it disappeared completely and could no longer be observed, even with a daily dose of 3.0 g. Weight of the animal: 4940 g.

14. Octatrienic amide (XI) Was fed, as described above, twice daily in amounts of 1.0 to 2.0 g. Exitus occurred after a total amount of 14.0 g. Weight of the animal: 2890 g. Liver granulated (white), stomach wall shows

numerous blood nodules, stomach content is dark red-brown.

15. Decatetraenic-amide (XV). The finely powdered amide was stirred with water and fed in amounts of 1 g twice a day. The substance had no visible effects on the organism in this concentration. Weight of the animal:2930 g.

III. Isolation and identification of dicarboxylic acids and dicarboxylic amide acids from the urine.

The isolation of the oxidation products was insofar simple as they represent almost exclusively substances which are difficultly soluble in water.

The alkaline reacting urine, which was collected under toluene, was collected for another 2 days after the last feeding, then filtered off from the sediments and evaporated in the vacuum (15 mm) to 100-200 cc. After cooling, the salts were removed by centrifuging and the clear liquid was acidified with 2 n -hydrochloric acid. (Congo), and left standing for a few hours in the refrigerator. The dicarboxylic acid or its derivative, which were already present in crystalline form in many cases, were filtered off and purified by treating the ammonium salts (dissolved in 2 n-ammonia) with adsorbents, like carbo medicinalis "Merck," frankonite KL, floridine XS and floridine XXF. Great caution is indicated, since the dicarboxylic-amide acids and the muconic anilide acids can be completely decomposed, particularly when carbo medicinalis "Merck" is used. In some cases prolonged boiling with water in the presence of the said decolorent is sufficient to convert the highly unsaturated decarboxylic acids into products that no longer crystallize.

1. 15 g sorbic acid yielded 0.200 g muconic acid (crude product, melting point 250 - 260 degC) which was converted into the dimethyl ester for identification. (27). Yield of pure ester: 38 mg.

3.810,3.802 mg substance: 5.10, 5.08 cc n/100 Na-OH.

C6H604 (142) calc. equiv. weight 71 found: equiv. weight 74,75.

In a second test we obtained from 30 g sorbic acid 220mg muconic acid with a defined melting point, from which we obtained 90 g pure dimethyl ester.

The alcoholic mother liquor obtained in the preparation of the ester had an intensive sorbic-methyl-ester odor.

Melting point of the muconic acid isolated from the urine 299 - 303 degC (corr.theory). Melting point of the muconic dimethyl ester: 157 degC. Eutectic melting point with a synthetic preparation: 156 degC. Yield of dicarboxylic acid from urine (related to the dimethyl ester obtained): 0.17 and 0.19% resp. of the theory.

2. After feeding 22 g sorbic methyl ester, no muconic acid could be isolated from the urine.

3. 30 g sorbic ethyl ester yielded 145 mg muconic acid (0.5% of the theory, melting point 300 degC (corr. theory).

3.401, 3.420 mg substance: 4.76, 4.80 cc n/100 NaOH.

C6H604 (142) cal. equiv. weight: 71 found equiv. weight: 71.4, 71.2

4. From 7.6 g sorbic amide we obtained 3.1 g muconic amide acid (33.2% of theory). The muconic amide acid crystallizes from water in colorless, shining needles which melt at 276-277 degC = 281 - 282 degC (corr. theory) under decomposition. 100 cc m water dissolve at 4 deg. C 0.0129 g muconic amide acid. Difficulty soluble in hot glacial acetic acid, insoluble in alcohol.

6.206 mg substance 4.39 cc n 100 NaOH. - 1.500 mg substance in 1.0 cc n/100 Na2CO3 with 8.3 mg platinum oxide after 20 min. end value. - 4.577 mg substance: 0.385 cc N2 (23 deg., 751 mm) - 4.183 mg substance: 7.820 mg So2, 1.835 mg H20.

C6H703N (141)	cal. equiv. weight:	141	F	2.00	C 51.06	H 4.96	N 9.92
	Found " "	141	F	2.03	50.98	4.91	9.59

Muconic amide acid reacts with PC15 much slower than muconic acid and yields after addition of absolute methanol trans.-trans-muconic dimethyleester, which melts at 157-157.5 degC (corr. theory). PC15 melts the acid amide group to chlorimide, which passes over into the ester over the methoxy-imide compound under the action of methanol.

4.060, 4.227 mg substance: 8.400, 8.780 mg CO_2, 2,215 , 2.340 mg H_2O

$C_8H_{10}O_4$ (170.1)	calc.	C 56.43	H 5.93
	found	56.43; 56.65	6.10; 6.19

Saponification of the muconic amide acid. 100 mg muconic amide acid were heated on the steambath for 90 minutes with 5 cc 30% alcoholic potash dye and 10 cc water, acidified after cooling with 2 n-hydrochloric acid, and filtered. The precipitate was dissolved in 2 n-soda lye, treated hot with animal charcoal, filtered off the filtrate was acidified and the muconic acid was precipitated in snow-white crystals. After standing in the cold for some time, the filtrate was sucked off, washed thoroughly with water, and dried over phosphorus pentoxide, Melting point 299deg. (corr.theory).

5.961, 5.295 mg substance: 8.45, 7.40 cc n/100 NaOH.

C6H604 (142). calc. equiv. weight 71 found equiv. weight 70.5, 71.5

5. From 19.5 g sorbicmethyl amide we obtained 9.2 g (43.8% of theory) muconic-methyl amide acid with a melting point of 217 degC (corr. theory) under decomposition. Muconic methyl amide acid dissolves in hot water, in large amounts of hot alcohol, in hot methanol, in pyridine, and is insoluble in ether, chloroform, and difficultly soluble in acetic ester.

4.186, 4.171 mg substance 8.335, 8.33 mg CO_2, 2.205, 2.155 mg H_2O. 5.600, 5.396 mg substance: 3.61, 3.47 cc n/100 NaOH. - 4.001, 3.945 mg substance: 0.321 (23 deg. 754 mm), 0.311 (22 deg., 755 mm) cc N_2.

C7H903N (155)	calc. equiv. weight	155	H 5.80	N 9.03	C 54.19
	Found " "	155	5.90	9.18	54.31
	" " "	155	5.78	9.06	54.46

6. 20 g sorbic anilide yielded 2.7 g muconic anilide acid (11.5% of theory); in a second test we obtained from 18 g sorbic anilide7.5 g muconic anilide acid (36% of theory) and in a third test 3.1 g muconic anilide acid (15.7% of theory) were isolated from the urine after feeding 17 g sorbic anilide. The muconic anilide acid is always precipitated in amorphous form from alkaline solution with diluted hydrochloric acid. The precipitate passes over into the crystalline state, however, when the aqueous suspension is heated. 3000 parts boiling water dissolve 1 part muconic anilide acid; it is resistant to boiling

concentrated hydrochloric acid, in which it dissolves and from which it is precipitated again on cooling. From hot water the substance crystallizes in irregular platelets which melt at 261-263 degC (corr. theory) under decomposition, brown coloration, and without sublimating. The amorphous muconic anilide acid, however, sinters (after drying in the vacuum over phosphorus pentoxide) at 250 degC. decomposes at 310 degC, and yields a sublimate of yellow-gold needles. Muconic-anilide acid is difficultly soluble in water, benzene, soluble in acetic ester, moderately soluble in absolute alcohol, easily soluble in methanol, insoluble in ether and chloroform.

3.945 mg substance: 9.545 mg CO_2, 1.85 mg H_2O. - 8.065 mg substance: 3.70 cc n/100 NaOH 3.656 mg substance: 0.212 cc N (20 deg. 741 mm)

$C_{12}H_{11}O_3N$ (217.1)	calc. equiv. weight:	217.1	C 66.32	H 5.11	N 6.45
	found " "	217	65.99	5.24	6.59

7. 2.5 g beta-methyl-sorbic amide yielded after feeding 1.91 g (62% of theory) beta-methyl-muconic amide-acid, melting point 269-261 degC (corr. theory). 1000 parts boiling water dissolve 4.1 parts beta-methyl-muconic-amide acid. It crystallized from water in long, fine, colorless prisms. The new substance is soluble in hot methanol, difficultly soluble in hot alcohol and in water, hardly soluble in acetic ester and in chloroform.

3.963, 4.134 mg substance 7.865, 8.180 mg CO_2, 2.080, 2.145 mg H_2O. 5.238, 5.300 mg substance: 3.37, 3.42 cc n/100 NaOH. - 3.894, 3.870 mg substance 0.312 (22 deg. 744 mm) 0.311 (21 deg., 742 mm) cc N.

$C_7H_9O_8N$ (155).

calc. equiv. weight:	155	C 54.19	H 5.80	N 9.03
Found " "	155; 155	54.13; 53.92	5.87;5.80	9.08;9.11

<u>Saponification of beta-methyl-muconic amide-acid</u>. 100 mg beta-methyl-muconic-amide acid were heated for 60 minutes on the steambath with 5 cc 30% aqueous potash lye, acidified after cooling with 2 n-hydrochloric acid, and a snow-white precipitate was obtained which was filtered off, dried and weighed. Yield 79.6 mg (79.6% of theory). Recrystallized from water. Melting point of the beta-methyl-muconic acid thus obtained 231-232 degC (corr. theory).

Eutectic melting point with a synthetic preparation (28).

231 - 232 degC (corr. theory).

4.190, 4.128 mg substance: 5.40, 5.37 cc n/100 NaOH

C7H804 (156) calc. equiv. weight: 78 found equiv. weight: 77.5, 78.7

8. After feeding 6.3 g octatrienec acid and 8.0 g decatetraenic acid resp. no dicarboxylic acid could be isolated from the urine.

9. 14.0 g octatrienic acid amide yielded 7.123 g (42% of theory) hexatriene (2,4,6)-dicarboxylic (1,6)-amide acid-(1). When the substance is precipitated with diluted hydrochloric acid from alkaline solution, we obtain fine, star-shaped needles. The hexatriene-dicarboxylic amide acid crystallizes from large amounts of boiling water in colorless, irregular hexagonal platelets which decompose at 263 deg. (corr. theory) (introduced into the block at 190 degC) without melting. Hexatriene-dicarboxylic-amide acid(XII) is soluble in hot water and diluted alkali, insoluble in acetic ester, alcohol, chloroform, carbon disulfide and conc. hydrochloric acid.

4.504 mg substance: 8.53 mg CO2, 2.00 mg H20.- 6.404 mg substance: 3.77 cc n/100 NaOH. - 4.272 mg substance: 0.310 cc n/100 N (19 deg., 742 mm).

C8H903N (167)	calc. equiv. weight:	167,	C 57.48	H 5.38	N 8.37
	found " "	170	57.38	5.52	8.28

Saponification of hexatriene-(2,4,6)-dicarboxylic-(1,6)-amide acid (I)

1.0 g amide acid (XII) were mixed with 10 cc 30% aqueous potash lye and heated for 2 hours on the steambath, acidified after cooling with hydrochloric acid, and the precipitate was filtered off and dried. Yield: 0.60 g (60 % of theory). The sharply dried hexatriene-1,6-dicarboxylic acid (XIII) was converted as usual with phosphorus pentachloride and methanol into the dimethyl ester. Melting point of the hexatriene-1,6-dicarboxylic-dimethyl ester thus obtained 169.5 - 170.5 degC (corr. theory); eutectic melting point with a synthetic reference preparation (29) 169.5 - 171 degC (corr. theory).

4.128, 4.013 mg substance: 9.245 mg CO2, 2.28, 2.20 mh H20

$C_{10}H_{12}O_4$ (196.1)	calc.	C 61.19	H 6.17
	found	61.08; 60.96	6.18; 6.13

10. From 7.1 g decatetraenic acid amide we obtained 1.7 g (20% of theory) octatotraene (2,4,6,8) -dicarboxylic-(1,8)-amide acid(1), which formed from water granular yellow crystals. The alkaline solution of the octatetraene-dicarboxylic-amide acid (XVI) fluoresces bluish-green in front of the analytical lamp. The not quite pure preparation melted under decomposition at 258 deg C (c. theory).

4.945 mg substance: 0.311 cc N (24 deg., 751 mm)

$C_{10}H_{11}O_8N$ (193.1) calc. N 7.25 found N 7.12

Saponification of octatetraene-(2,4,6,8)-dicarboxylic-(1,8)-amide acid(I)

800 mg amide acid (XVI) were mixed with 10 cc 30% aqueous potash lye and heated for 2½ hours on the steambath. Acidified with hydrochloric acid after cooling, filtered off from the precipitate and dried sharply. Yield: 0.440 g. The dry octatetraene-1,8-dicarboxylic acid was converted with 0.8 g phosphorus trichloride and methanol as usual into the dimethyl ester. Yield: 0.240 g. Recrystallized from glacial acetic acid the dimethyl ester melted at 212 degC (corr. theory). Eutectic melting point with a synthetic reference preparation (29) 213 degC (corr. theory).

3.884 mg substance: 9.270 mg CO_2, 2.200 mg H_2O.

$C_{12}H_{14}O_4$ (222.1) calc. C 64.84 H 6.35 Found: C 65.09 H 6.34

11. Because of the high toxicity of the crotonic amide, only small quantities could be fed. It was not possible to isolate oxidation products in pure form from the urine. The crystalline decomposition products obtained in traces in the preparation of the urine were not sufficient for an accurate investigation.

12. 21 g crotonic anilide yielded after feeding 3.2 g (14% of the theory) N-crotonyl-p-amino-phenol with a defined melting point. The precipitate obtained in the evaporation of the alkaline dark-colored urine was centrifuged and recrystallized from acetone after drying. When the acetone solution is decolorized with animal charcoal, the new substance crystallizes in coarse, snow-white prisms which dissolve in acetone, alcohol, hot water, boiling acetic

ester, glacial acetic acid, methyl-ethyl-ketone and in pyridine, and which are insoluble in ether, petroleum ether, chloroform and benzene. From the acetone-mother liquor we obtained after concentration in addition 150 mg pure p-amino-phenol which melts at 189.5 - 190.5 degC (corr. theory). The N-crotonyl-p-aminophenol, dissolved in diluted methanol, yields a good green iron tri-chloride reaction. Eutectic melting point with the synthetic preparation: 189.5 - 190.5 degC.

1.690 mg substance in 20 cc glacial acetic acid with 10.3 mg palladium (II)-oxide after 3 minutes end value.

4.207, 3.890 mg substance; 10,435, 9.65 mg CO_2, 2.37, 2.225 mg H_2O.

$C_{10}H_{11}O_2N$ (177.1)	calc.	= 1.00	C 67.76	H 6.27
	found	= 1.06	67.61; 67.65	6.30;6.40

For conversion into the N-butyryl-p-aminophenol, 800 mg N-crotonyl-p-aminophenol were dissolved in 70 cc acetic ester and 20 cc alcohol, mixed with 500 mg palladium (II)-oxide and hydrated until the hydrogen absorption stopped. After filtering off the palladium, the solvent was evaporated in the vacuum and the residue recrystallized from acetone. $FeCl_2$-reaction in aqueous methanol green. The N-butyryl-p-aminophenol crystallized from acetone in regular, hexagonal glass-clear platelets, from water in densely feathered needles. Melting point 138 - 139 degC (corr. theory). Eutectic melting point with the synthetic preparation 138 to 139 degC (corr theory). The substance is easily soluble in acetone, acetic ester, hot water and methanol, soluble in ether, insoluble in petroleum ether.

3.915, 4.004 mg substance, 9.61, 9.86 mg CO_2; 2.525,2.58 mg H_2O.

$C_{10}H_{13}O_2N$ (179.1)	calc.	C 67.00	H 7.32
	found	66.94;67.16	7.21; 7.21

13. 17 g beta, beta-dimethyl-acrylic amide (III) yielded 10.1 g (43% of theory) measconic-alpha-amide acid which was already described by R. Anschuetz (31). It is soluble in hot water, in hot alcohol, insoluble in cold water and ether. From hot water the megaconic alpha amide acid crystallizes in colorless coarse crystals which melt at 223 degC (corr. theory). R. Anschuetz indicates

222 degC.

4.137 mg substance: 7.100 mg CO_2; 2.065 mg H_2O. - 5.750, 5.458 mg substance: 4.42, 4.17 cc n/100 NaOH. - 4.140 mg substance: 0.386 cc N (21 deg. 751 mm).

$C_5H_7O_3N$ (129)	calc. equiv. weight;	129	C 46.51	H. 5.42	N 10.85
	found " "	130; 130	46.80	5.59	10.70

14. After feeding 13 g 5-methyl-pyromucic amide (VI), we isolated 5.1 g (32% of theory) furane-dicarboxylic-(2,5)-amide acid- (2). This substance was described by I. Phelps and W. Hale (32) with a melting point of 280-281 (corr.). It is soluble in hot water, insoluble in alcohol and difficultly soluble in glacial acetic acid. We isolated the furane-dicarboxylic-(2,5)-amide acid-(2) as a dimorphous substance which crystallizes from glacial acetic acid in magnificant long silky needles and which is separated from water in the form of compact prisms and in the form of long fine needles. The crystallization tendency of the prisms is greater than that of the needles. Separation by fractioned crystallization. Melting point of the prisms 283 degC (Corr.theory).

4.125 mg substance: 7.025 mg CO_2, 1.195 mg H_2O.- 7.002, 8.549 mg substance: 4.50, 5.57 cc n/100 NaOH. - 4.535 mg substance: 0.359 cc N (23 deg. 751 mm).

$C_3H_5O_4N$ (155)	calc. equiv. weight:	155	D 46.45	H 3.22	N 9.03
	found " "	155;154	46.45	3.24	9.02

Melting point of the needles 284degC (corr. theory).

4.546 mg substance: 7.755 mg CO_2, 1.38 mg H_2O. - 6.861 mg substance: 4.54 cc n/100 NaOH. - 4.894 mg substance: 0.386 cc n/100 N (22 deg 748 mm).

found equiv. weight: 15 C 46.52 H 3.40 N 8.98

<u>Saponification of furane-dicarboxylic-(2,5)-amide acid-(2)</u>. 540 mg amide acid (VII) were mixed with 15 cc 30% alcoholic potash lye and heated for 60 minutes on the steambath; the dipotassium salt separated in the course of the saponification was dissolved in water, then evaporated from the alcohol, the residue was acidified with 2 n-hydrochloric acid and the separated colorless

crystals were filtered off. The furane-dicarboxylic acid-(2,5) was dried sharply and introduced into excess methanol which had been saturated at -10 degC with dry hydrochloric acid gas. After heating for 60 minutes under reflux it was concentrated on the steambath, then cooled and a crystal sludge was separated which was filtered off and recrystallized from water. Melting point of the furance-dicarboxylic-(2,5)-dimethyl ester 109 degC (corr. theory). See P. Yoder and B. Tollens (33) as well as L. Phelps and W. Hale (34).

15. 20 g beta-methyl-furyl-(2)-acrylic amide yielded 20.1 g (83% of theory) furyl-acrylic amide-(2)-carboxylic acid-(5) crude product or 15.4 g (64% of theory) pure product. The new substance (IX) crystallizes from water in colorless prisms with beautiful twin formations or, when slowly precipitated, in long hair-shaped needles. Melting point 280 degC (corr. theory) under decomposition. Soluble in large amounts of hot water, in hot methanol, in alcohol, difficultly soluble in acetic ester, insoluble in ether.

4.208, 4.053 mg substance: 8.240, 7.945 mg CO_2; 1.505, 1.445 mg H_2O.- 7.391, 7.297 mg substance: 4.14, 4.04 cc n/100 NaOH. - 4.364, 4.141 mg substance: 0.293 (21 deg. 761 mm) 0.272 (20 deg. 756 mm) cc N.

$C_8H_7O_4N$ (181).

calc. equiv. weight:	171	C 53.03	H 3.86	N 7.73
found " "	178,180	53.40; 53.46	4.00; 3.99	7.83;7.61

IV. Syntheses

1. N-crotonyl-aminophenol 10.4 g freshly distilled crotonic chloride were taken up with 100 cc ether dry and mixed at room temperature under vigorous shaking with an ether solution of 21.8 g p-aminophenol. After a few minutes no acid chloride could be smelled anymore. We left the reaction mixture standing for 2 hours, then filtered off the precipitate and dissolved out the p-aminophenol hydrochloride on the suction filter with greatly diluted hydrochloric acid. The crude product was already very pure. Yield 17.6 g (99.4% of theory). After recrystallization from water the N-crotonyl-p-aminophenol melted at 189-190 degC (corr. theory). For solubility and crystal form see p. 20.

4.033 mg substance: 10.01 mg CO_2; 2.20 mg H_2O.

$C_{10}H_{11}O_2N$ (177.1)	calc.	C 67.76	H 6.27
	found	67.69	6.10

2. N-butyryl-p-aminophenol 17.6 g butyric acid were mixed with 50 cc dry benzene and 24 g freshly distilled thionyl chloride and heated on the steam-bath under the reflux condenser until the formation of hydrochloric acid stopped. The reaction mixture was then distilled with a fractioning attachment and 5.0 g unreacted butyric acid were recovered. Yield 10.5 g (59.3% of theory). Bp-750 = 100 to 101 degC.

To an ether solution of 16.5 g p-aminophenol were added 9.0 g butyric chloride. The mixture was shaken thoroughly and the reaction product was filtered off after standing for 2 hours. The hydrochloride was dissolved by treating the precipitate with water. Yield of N-crotonyl-p-aminophenol 9.3 g (67% of theory). The residue was dissolved in acetone, heated with animal charcoal, filtered. The solvent was evaporated and then recrystallized from water, yielding beautifully feathered neddles. Melting point 139-140 deg C (corr. theory).

4.114 mg substance: 10.11 mg CO_2; 2.66 mg H_2O.

$C_{10}H_{18}O_2N$ (179.1)	calc.	C 67.00	H 7.32
	found	67.02	7.23

Footnotes

(1) Paper delivered before the District Group Frankfurt a.Main of the Association of German Chemists in Hoechst a.M. on Nov. 12, 1936. Review: Z. angew. Chem. 49,928 (1936).

(2) R. Kuhn and M. Hoffer, Ber. chem. Ges. 65, 655 (1932)

(3) See R. Kuhn and K. Livada, This journal 220, 235 (1933); R. Kuhn, F. Koehler and L. Loehler, this journal 242,171 (1936)

(4) This journal 159,297 (1926).

(5) Moerner, this journal 13,12 (1889); M. Jaffe and P. Hilbert, this journal 12,295 (1888).

(6) Liebigs Ann. 353, 175 (1907)

(7) Ber. chem. ges. 68, 947 (1935)

(8) R. Kuhn, F. Koehler and L. KOehler, this journal 242, 171 (1936)

(9) Liebigs Ann. 353, 139 (1907)

(10) Z. physik. Chem. 29, 378 (1935)

(11) Klin. Wchschr. 15,1819 (1916); Ber. Physiol. 96, 654 (1937); this hournal 246, 124 (1937).

(12) R. Kuhn, and Ch Grundmann, Ber. chem. Ges. 69, 1757 (1936)

(13) P. E. Verkade, Biochemie J. 28, 31 (1934); this journal 225, 230 (1933)

(14) B. Flaschentraeger and K. Bernhard, this journal 238, 221 (1936)

(15) Ber. chem. Ges. 33, 2140 (1900)

(16) Ber. chem. Ges. 34, 2221 (1901)

(17) Chem. Z. 1928, I, 2207

(18) Ber. chem. Ges. 65, 655 (1932)

(19) Ber. chem. Ges. 64, 1978 (1931)

(20) Benchem. Ges. 69, 98 (1936)

(21) Ber. chem. Ges. 63,2164 (1930

(22) Ber. chem. Ges. 47, 1786 (1914)

23) Ber. chem. Ges. 44, 1636 (1911)

24) J. Amer. Chem. Soc. 95, 1955 (1909)

(25) See organic Syntheses 14,62 (1934)

(26) We are indebted to Mr. H. Ruelius, cand. chem. for his assistance who made comparison tests about thesynthesis of this acid. The Perkin synthesis with 5-methyl-furfurol yields much poorer yields than the above described synthesis by Doebner (70% of theory), both with potassium acetate (24% of theory) and with sodium acetate (15% of theory).

(27) R. Kuhn and Ch. Grundmann, Ber. chem. Ges. 69, 1757 (1936)

(28) R. Kuhn and Ch. Grundmann, Ber. chem. Ges. 69,1757 (1936)

(29) R. Kuhn and Ch. Grundmann, loc. cit.

(30) R. L. Shriner and R. Adams, J. Amer. Chem. Soc. 46,1683 (1924)

(31) Liebigs Ann. 353, 175 (1907)

(32) Amer. Chem. J. 25,453 (1901); Chem. Z. 1901, I, 272

(33) Ber. chem. Ges. 34, 3453 (1902)

(34) Amer. chem. J. 25, 452 (1901)

(Translated by Carl Demrick Associates,Inc/IE/t)

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Einwage	0,7749 g
Verbraucht n/100-Thiosulfat	8,8 ml
Laminarin, berechnet	0,2 %

Zusammenfassung.

Reine Präparate von Fucoidin können durch Sammeln der beim Liegen von Laminariablättern sich in Rissen bildenden wasserklaren Tropfen erhalten werden.

Das Fucoidin ist ein Polymeres eines Kohlenhydrat–Schwefelsäureesters von der allgemeinen Zusammensetzung:

$$R—OSO_2O—Me.$$

R besteht zu 60 % aus Fucose. Me ist hauptsächlich Natrium, etwas Kalium und geringe Mengen Calcium und Magnesium.

Literatur.

1. Hassid, J. Amer. Chem. Soc. 55, 4168 (1933).
2. Bird u. Haas, Biochemic. J. 25, 403 (1931).
3. Butler, Biochemic. J. 28, 759 (1934).
4. Haas u. Russell-Wells, Biochemic. J. 17, 696 (1923).
5. Dieselben, Biochemic. J. 23, 425 (1929).
6. Hoagland u. Lieb, J. of Biol. Chem. 23, 287 (1915).
7. van der Haar, Anleitung zum Nachweis, Trennung und Bestimmung der Monosaccharide und Aldehydsäuren, Berlin 1920.
8. Kullgreen u. Tydén, Ingeniörs-Vetenskapsakademiens Handlingar, Nr. 94, 1929.
9. Kylin, Diese Z. 83, 171 (1913).
10. Derselbe, Diese Z. 94, 337 (1915) (vgl. S. 404 ff.).
11. Nelson u. Cretcher, J. of Biol. Chem. 94, 147 (1931).
12. Takahashi u. Shirahama, Bull. agricult. chem. Soc. Japan 8, 8 (1932).

Über die biologische Oxydation hochungesättigter Fettsäuren.

Ein neuer Weg zur Darstellung von Polyen-dicarbonsäuren[1]).

Von

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(Der Schriftleitung zugegangen am 6. Mai 1937.)

Nachdem hochungesättigte Fettsäuren, die aliphatischen Polyen-carbonsäuren (I)

$$\text{I} \quad H_3C—(CH=CH)_n—COOH$$

synthetisch zugänglich geworden sind[2]), haben wir Versuche über ihr Verhalten im Tierkörper angestellt. Es interessierte dabei besonders der Vergleich mit den gesättigten Fettsäuren, die auch in Form ihrer Ester und Glyceride schon so oft Gegenstand der Untersuchung gewesen sind. Man konnte erwarten, daß bei geeigneter Kettenlänge die Polyen-carbonsäuren, den Fettsäuren nach Verkade entsprechend, in Polyen-ω,ω'-dicarbonsäuren (II) übergehen würden,

$$\text{II} \quad HOOC—(CH=CH)_n—COOH,$$

von denen zu Beginn unserer Untersuchung kein höheres Glied als die Muconsäure (n = 2) bekannt war.

Wir konnten in der Tat nach Fütterung von Sorbinsäure (I, n = 2) an Kaninchen aus dem Harn *trans, trans*-Muconsäure isolieren, die in den Dimethylester vom Schmelzp. 157° (Mischprobe) übergeführt wurde. Die Ausbeute (0,1—0,2 %) war jedoch sehr gering. Aus Sorbinsäure-*methylester* wurde überhaupt keine Dicarbonsäure erhalten, aus Sorbinsäure-*äthylester* erhielten wir 0,5 %. Das Bild änderte sich wesentlich als wir zu stickstoffhaltigen Derivaten übergingen. Aus dem Harn von

[1]) Vorgetragen vor dem Bezirksverein Frankfurt a. M. des Vereins Deutscher Chemiker in Höchst a. M. am 12. November 1936. Referat: Z. angew. Chem. 49, 928 (1936).

[2]) R. Kuhn u. M. Hoffer, Ber. chem. Ges. 65, 655 (1932).

Kaninchen, die mit Sorbinsäure-*amid* gefüttert waren, isolierten wir in einer Ausbeute von 32 % d. Th. eine in glänzend weißen Nädelchen krystallisierende Verbindung $C_6H_7O_3N$ vom Schmelzpunkt 281—282°. Diese hat sich als die noch unbekannte Muconamidsäure HOOC—CH=CH—CH=CH—CO·NH_2 erwiesen. Sie ließ sich durch alkoholische Kalilauge unter Abspaltung von Ammoniak glatt zu *trans, trans*-Muconsäure, Schmelzp. 299° (Mischprobe), verseifen. Noch ergiebiger verlief die ω-Oxydation von Sorbinsäure-*methyl-amid*, aus dem wir 44 % d. Th. reine Muconmethyl-amidsäure HOOC—CH=CH—CH=CH—CO·NH·CH_3 vom Schmelzp. 217° erhielten. Aus Sorbinsäure-*anilid* entstand mit Ausbeuten bis zu 36 % d. Th. die bei 263° schmelzende Mucon-anilidsäure HOOC—CH=CH—CH=CH—CO·NH·C_6H_5.

Die Oxydation der endständigen Methylgruppe wird durch Überführung der Polyen-carbonsäure in ihr Amid, Methylamid, Anilid usw. außerordentlich begünstigt. Die Ausbeuten sind so gute, daß man das Kaninchen für die präparative Darstellung mancher sonst kaum erhältlichen Verbindungen heranziehen wird. Aus β-Methyl-sorbinsäure-amid erhielten wir z. B. in einer Ausbeute von 62 % d. Th. die β-Methylmucon-amidsäure HOOC—CH=CH—C(CH_3)=CH—CO·NH_2 in langen Prismen vom Schmelzp. 259—261°. Wollte man diese Substanz von der β-Methyl-muconsäure ausgehend darstellen, so wäre es nicht leicht, die Amidgruppe nur an das der Methylgruppe benachbarte Carboxyl zu bringen.

Maßgebend für die hohen Ausbeuten erscheint uns

1. die Unterdrückung der β-Oxydation durch die Amidbildung der Carboxylgruppe,

2. die schon ohne Amidierung erkennbare Herabsetzung der Verbrennungsgeschwindigkeit durch die konjugierten Doppelbindungen,

3. die weitere Hemmung der β-Oxydation, welche die in manchen Fällen vorliegende β-ständige Methylgruppe (β-Methylsorbinsäure) bewirkt[1]).

Der Einfluß der Amidbildung ist überraschend. B. Flaschenträger[2]) fand nämlich bei der Fütterung an Hunde, daß die Sebacinsäure HOOC—(CH_2)$_8$—COOH zu 61 % unverändert im

[1]) Vgl. R. Kuhn u. K. Livada, Diese Z. 220, 235 (1933); R. Kuhn, F. Köhler u. L. Köhler, Diese Z. 242, 171 (1936).

[2]) Diese Z. 159, 297 (1926).

Harn ausgeschieden wurde, die Sebamidsäure HOOC—(CH_2)$_8$—$CONH_2$ aber nur zu 10 %. Das Amid war also leichter abgebaut worden als die Säure. In unseren Fällen trifft das Gegenteil zu.

Nach Verfütterung von Crotonsäure-anilid $C_{10}H_{11}ON$ ließ sich aus dem Harn weder Malein- noch Fumar-anilidsäure isolieren. Wir erhielten vielmehr in einer Ausbeute von 14 % d. Th. eine bei 189—190° schmelzende Verbindung $C_{10}H_{11}O_2N$, die also 1 Sauerstoffatom mehr enthielt, als das Ausgangsmaterial. Bei der gelinden katalytischen Hydrierung wurden 1,06 Mole H_2 aufgenommen. Der Dihydrokörper $C_{10}H_{13}O_2N$ schmolz bei 138 bis 139°. Die Verbindung $C_{10}H_{11}O_2N$ gab in Methanol mit Ferrichlorid Grünfärbung, war in Natronlauge löslich und gab sich so als Phenol zu erkennen. Da Acetanilid und andere Derivate des Anilins im Tierkörper zu Derivaten des p-Aminophenols oxydiert werden[1]), war zu vermuten, daß die aus dem Harn isolierte Substanz N-Crotonyl-p-aminophenol H_3C—CH=CH—CO—NH—C_6H_4—OH darstellt. Die Synthese dieser Substanz aus p-Amino-phenol und Crotonsäure-chlorid hat die Richtigkeit der Erwartung erwiesen (Mischprobe). Die Dihydroverbindung $C_{10}H_{13}O_2N$ stimmt in allen Eigenschaften mit dem von uns aus Buttersäure-chlorid und p-Amino-phenol erhaltenen N-Butyryl-p-amino-phenol H_3C—CH_2—CH_2—CO—NH—C_6H_4—OH überein. Hier liegt der einzige Fall der Ausscheidung eines p-Aminophenol-derivates vor, der uns bei Fütterungsversuchen mit Aniliden im Laufe der vorliegenden Untersuchung begegnet ist. Als Anfangsglied in der homologen Reihe der Polyen-carbonsäuren nimmt die Crotonsäure auch biologisch eine Ausnahmestellung ein.

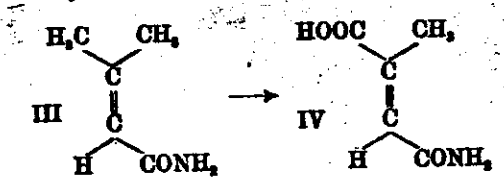
Das Amid der β,β-Dimethyl-acrylsäure (H_3C)$_2$C=CH—$CONH_2$ lieferte in einer Ausbeute von 43 % d. Th. eine Carbonsäure $C_5H_7O_3N$ vom Schmelzp. 223°, die sich als identisch mit der von R. Anschütz[2]) beschriebenen Mesacon-α-amidsäure (Schmelzp. 222°) erwiesen hat. Dieser Befund ist von stereochemischem Interesse, da durch Methyloxydation der β,β-Dimethylacrylsäure nicht nur Mesaconsäure (*trans*-Form) sondern auch Citraconsäure (*cis*-Form) entstehen kann. Bei der Oxydation im Tierkörper wird anscheinend nur diejenige der beiden Methylgruppen angegriffen, die zur Carbonamidgruppe *trans*-ständig ist.

[1]) K. Mörner, Diese Z. 13, 12 (1889); M. Jaffé u. P. Hilbert, Diese Z. 12 295 (1888).

[2]) Liebigs Ann. 353, 175 (1907).

In diesem Zusammenhang ist daran zu erinnern, daß auch im Campher bei der Bildung von π-Oxycampher [Y. Asahina und M. Ishidate[1]] von den beiden Methylen an der Brücke nur dasjenige vom Organismus oxydativ angegriffen wird, das zur Carbonylgruppe *trans*-ständig ist. Sollte hier eine allgemeinere Gesetzmäßigkeit vorliegen, so wäre es möglich, in schwierigen Fällen neuartige Anhaltspunkte bei der Zuordnung von cis-trans-Formeln zu gewinnen. So wie aus der Dimethyl-acrylsäure können auch aus der Geraniumsäure, je nachdem welches der beiden Methyle im endständigen Isopropyliden-rest vom Tierkörper zum Carboxyl oxydiert wird, zwei verschiedene *cis-trans*-isomere Hildebrandt-säuren hervorgehen. Welche von diesen in der aus dem Harn isolierten Verbindung vom Schmelzp. 194° vorliegt, konnten wir in unserer letzten Arbeit[2]) nicht sagen. Die vorliegenden Ergebnisse machen es wahrscheinlich, daß es sich um die *trans*-Form handelt.

Die Konstitution der Mesacon-α-amidsäure hat R. Anschütz[3]) aus langen, mühevollen Untersuchungen erschlossen. Der Kernpunkt war die Entscheidung, welche der beiden Estersäuren $HOOC—C(CH_3)=CH—COOCH_3$ (α) und $H_3COOC—C(CH_3)=CH—COOH$ (β) bei 52° bzw. 84° schmilzt. Die Schlußfolgerung, daß in der niedriger schmelzenden α-Form die Methylgruppe der freien Carboxylgruppe benachbart ist, ergab sich aus einer vom γ-Dibrom-α-methyl-acetessigester ausgehenden Synthese. Die Richtigkeit der von R. Anschütz den α- und β-Verbindungen zuerteilten Formeln wird vom Kaninchen in einfachster Weise bestätigt: **die aus Dimethyl-acrylsäure-amid erhaltene Mesacon-α-amidsäure kann die Methylgruppe nur in Nachbarschaft zur *freien* Carboxylgruppe tragen.**

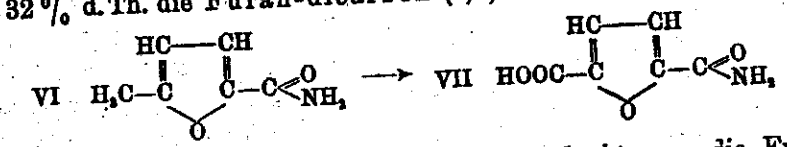


Auffallenderweise konnten aus β-Methyl-β-äthyl-acrylsäure V $(H_3C)(H_5C_2)C=CH—COOH$,

[1]) Ber. chem. Ges. 68, 947 (1935).
[2]) R. Kuhn, F. Köhler u. L. Köhler, Diese Z. 242, 171 (1936).
[3]) Liebigs Ann. 353, 189 (1907).

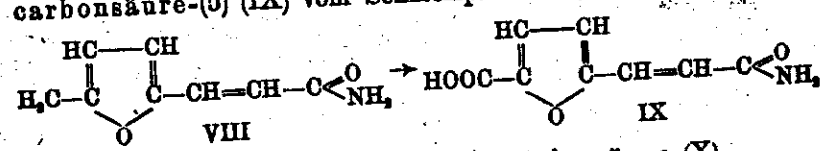
die sowohl als Äthylester wie als Amid (Schmelzp. 128—128,5°) gefüttert wurde, keine harnfähigen Carbonsäuren gewonnen werden.

Um so günstiger verliefen Versuche mit Polyen-carbonsäure-amiden, bei denen zwei konjugierte Doppelbindungen durch eine Sauerstoff-brücke zu einem Furanring zusammengefaßt sind. Aus einer Untersuchung von K. W. Hausser, R. Kuhn, A. Smakula und A. Deutsch[1]) war bekannt, daß in optischer Hinsicht (Lage der Absorptionsbanden) der Furanring einem offenen System von zwei konjugierten Doppelbindungen gleichwertig ist. Wir haben geprüft, ob diese Gleichwertigkeit auch biologisch für die Oxydationsvorgänge im Tierkörper zutrifft. Aus 5-Methyl-brenzschleimsäure-amid (VI) erhielten wir in einer Ausbeute von 32 % d. Th. die Furan-dicarbon-(2,5)-amidsäure-2 (VII) vom



Schmelzp. 284°. B. Flaschenträger[2]) verdankt man die Entdeckung, daß geringe Mengen von Furan-dicarbonsäure-(2,5), nämlich 3—5 mg je Tag, einen anscheinend normalen Bestandteil des menschlichen Harns bilden. Das von uns isolierte Amid (VII) wurde durch Verseifung mit alkoholischer Kalilauge in die Furan-α,α'-dicarbonsäure verwandelt, deren Dimethylester den richtigen Schmelzp. 109° zeigte.

Das β-[5-Methyl-furyl-(2)]-acrylsäure-amid (VIII) ergab die Rekord-ausbeute von 83 % d. Th. Furan-acrylamid-(2)-carbonsäure-(5) (IX) vom Schmelzp. 280°.



Das acyclische Analogon, die Octatriensäure (X)

X $H_3C—CH=CH—CH=CH—CH=CH—COOH$

lieferte, als Natriumsalz verfüttert, keine Dicarbonsäure. Ihr Amid (XI) führte jedoch zur Ausscheidung einer bei 263° unter Zersetzung schmelzenden, schwer löslichen, farblosen Carbonsäure

[1]) Z. physik. Chem. 29, 378 (1935).
[2]) Klin. Wschr. 15, 1819 (1936); Ber. Physiol. 96, 654 (1937); Diese Z. 246, 124 (1937).

$C_6H_9O_3N$, von der 42 % d. Th. erhalten wurden. Durch Verseifung mit wäßriger Kalilauge wurde daraus die Hexatrien-1,6-dicarbonsäure (XIII) gewonnen. Die aus dem Harn isolierte Verbindung stellt somit die Hexatrien-2,4,6-dicarbon-1,6-amidsäure-1 (XII) dar.

XI $H_2C—CH=CH—CH=CH—CH=CH—CONH_2$

XII $HOOC—CH=CH—CH=CH—CH=CH—CONH_2$

XIII $HOOC—CH=CH—CH=CH—CH=CH—COOH$

Die Hexatrien-1,6-dicarbonsäure (XIII) war bis vor kurzem unbekannt. Sie wurde vor einigen Monaten, ausgehend vom Oxalosorbinsäure-ester, erstmals synthetisch erhalten[1]). Zur sicheren Identifizierung führten wir die aus dem Harn erhaltene Dicarbonsäure mit Phosphorpentachlorid und Methanol in den Dimethylester über, der bei 171° schmolz und mit dem Hexatrien-1,6-dicarbonsäure-dimethylester von R. Kuhn und Ch. Grundmann (Schmelzp. 171,5°) keine Schmelzpunktserniedrigung zeigte.

Bei der Dekatetraensäure (XIV) ist das Natriumsalz

XIV $H_2C—CH=CH—CH=CH—CH=CH—CH=CH—COOH$

bereits so schwer löslich, daß wir die gleichfalls fast unlösliche freie Säure verfütterten. Aus dem Harn des Kaninchens war weder die Ausgangs-substanz noch eine Dicarbonsäure erhältlich. Als wir jedoch zum Amid (XV) übergingen, konnten wir durch Ansäuern des Harns in einer Ausbeute von 20 % d. Th. eine sehr schwer lösliche Carbonsäure gewinnen. Diese ist Octatetraen-2,4,6,8-dicarbon-1,8-amidsäure-1 (XVI), denn sie ließ sich durch wäßrige Kalilauge zur gelben Octatetraen-1,8-dicarbonsäure (XVII) verseifen.

XV $H_2C—CH=CH—CH=CH—CH=CH—CH=CH—CONH_2$

XVI $HOOC—CH=CH—CH=CH—CH=CH—CH=CH—CONH_2$

XVII $HOOC—CH=CH—CH=CH—CH=CH—CH=CH—COOH$

Der Konstitutionsbeweis beruht auch in diesem Falle auf dem Vergleich der Dimethylester. Der aus dem Harn erhaltene Octatetraen-1,8-dicarbonsäure-dimethylester schmolz bei 212°, das nach der Oxalo-ester-synthese von R. Kuhn und Ch. Grundmann[1]) erhaltene Präparat bei 213°. Der Mischschmelzpunkt lag bei 213°.

12 [1]) R. Kuhn u. Ch. Grundmann, Ber. chem. Ges. 69, 1757 (1936).

Vergleicht man die Ausbeuten an ω-Dicarbonsäuren, die einerseits aus Fettsäuren, anderseits aus Polyen-carbonsäuren erhältlich sind, hinsichtlich der Abhängigkeit von der Anzahl der Kohlenstoff-atome in der Kette, so ergibt sich: Aus den Fettsäuren erhält man die maximale Ausbeute an Dicarbonsäure bei 10 C-Atomen [Mensch[1])] bzw. bei 8 C-Atomen [Hund[2])], aus den Amiden der Polyen-carbonsäuren bei 8 C-Atomen (Kaninchen).

4 C-Atome:	Crotonsäure-amid	~ 0 % d. Th.
6 C-Atome:	Sorbinsäure-amid	32 % d. Th.
8 C-Atome:	Octatriensäure-amid	42 % d. Th.
10 C-Atome:	Decatetraensäure-amid	20 % d. Th.

Beim Abfall von der Trien- zur Tetraensäure ist zu berücksichtigen, daß die Ungunst der Löslichkeitsverhältnisse und damit sehr wahrscheinlich auch der Resorptionsverhältnisse mit steigender Zahl der konjugierten Doppelbindungen rasch zunimmt. Der starke Abfall von der Sorbinsäure zur Crotonsäure mag teilweise mit der beträchtlichen Giftigkeit des Crotonsäure-amids zusammenhängen, die zum Verlust vieler Kaninchen geführt hat, obwohl nur verhältnismäßig kleine Mengen verfüttert wurden. Wir möchten darauf hinweisen, daß noch einige weitere Amide, Methylamide und Anilide an den Kaninchen teilweise recht starke pharmakologische Wirkungen zeigten, worüber im Versuchsteil näher berichtet wird. Lähmungserscheinungen, aber auch Krämpfe der Extremitäten, meist an den Hinterläufen beginnend, standen dabei im Vordergrund. In manchen Fällen kam es zu völliger Narkose.

Beschreibung der Versuche.

I. Präparate.

1. Hexadien-(2,4)-säure-(1); Sorbinsäure (I, n = 2). Aus Crotonaldehyd nach O. Doebner[3]). Ausbeute an schmelzpunktsreiner Säure: 25 % d. Th., Schmelzp. 135° (k. Th.).

2. Hexadien-(2,4)-säure-(1)-methylester. Nach O. Doebner und A. Wolff[4]). Ausbeute 99 % d. Th., Siedep.$_{20}$ = 71°.

3. Hexadien-(2,4)-säure-(1)-äthylester[4]). Ausbeute 81 % d. Th., Siedep.$_{11}$ = 81,5°, n_D^{19} = 1,4942.

4. Hexadien-(2,4)-säure-(1)-amid[4]). Ausbeute 91 % d. Th., Schmelzpunkt 171,5—172,5° (k. Th.).

13 [3]) P. E. Verkade, Biochemic. J. 28, 31 (1934); Diese Z. 225, 230 (1933).
14 [2]) B. Flaschenträger u. K. Bernhard, Diese Z. 238, 221 (1936).
15 [3]) Ber. chem. Ges. 33, 2140 (1900).
16 [4]) Ber. chem. Ges. 34, 2221 (1901).

5. Hexadien-(2,4)-säure-(1)-methyl-amid. 52 g im Vakuum frisch destilliertes Sorbinsäure-chlorid wurden mit trockenem Äther verdünnt, mit Aceton-Kohlensäureschnee gekühlt und langsam mit einem geringen Überschuß von flüssigem Methylamin versetzt. Nach dem Abdunsten des Äthers wurde der Rückstand mit Aceton gut ausgekocht. Das durch Verdampfen des Acetons zur Abscheidung gebrachte Sorbinsäure-methylamid krystallisierten wir aus sehr verdünntem Alkohol um, wobei große, farblose Plättchen erhalten wurden. Ausbeute 50 g (100 % d. Th.), Schmelzpunkt 141° (k. Th., sublimiert). Die Substanz ist löslich in Aceton, Alkohol, Methanol und heißem Wasser.

4,167 mg Subst.: 10,30 mg CO_2, 3,40 mg H_2O. — 3,688 mg Subst.: 10,49 ccm N_2 (21°, 754 mm).

$C_7H_{11}ON$ (125,1)	Ber.	C 67,14	H 8,87	N 11,19
	Gef.	„ 67,41	„ 9,13	„ 10,49.

6. Hexadien-(2,4)-säure-(1)-anilid. Nach O. Doebner und A. Wolff[1]). Ausbeute 72 % d. Th., Schmelzp. 155—156° (k. Th.).

7. Methyl-(2)-hexadien-(2,4)-säure-(1)-amid; β-Methyl-sorbinsäure-amid. — a) Pentanol-(4)-on-(2). Wir gewannen diese Substanz nach den Angaben eines Patentes der I. G. Farbenindustrie [F.P. 626729[1])], fügten jedoch vor der Destillation etwas Hydrochinon zu, wodurch die Ausbeuten verbessert wurden. 300 g trockenes Aceton wurden mit 90 g Acetaldehyd versetzt. Unter kräftigem Kühlen mit Eis-Kochsalz-Mischung wurden allmählich zu dem gekühlten Reaktionsgemisch 6 g fein gepulvertes Ätzkali zugefügt, mit etwas Hydrochinon versetzt und unter Wasserabschluß über Nacht im Eisschrank stehen gelassen; hierauf mit Kohlensäure behandelt, vom abgeschiedenen Bicarbonat abfiltriert und fraktioniert. Siedep.$_{14}$ 60—70°. Rohausbeute 50 g (24 % d. Th. ber. auf Acetaldehyd).

b) Penten-(2)-on-(4); Äthyliden-aceton. Die Entwässerung des Pentanolons wurde mit 1½ Molen Essigsäureanhydrid und einer Spur Chlorzink durchgeführt. Wir erhitzten 60 Minuten im Ölbad auf 150—160° und erhielten nach der Aufarbeitung in gewöhnlicher Weise das Äthylidenaceton in einer Ausbeute von 49—52 % d. Th.

c) Methyl-(2)-hexadien-(2,4)-säure-(1). Der nach R. Kuhn und M. Hoffer[2]) erhaltene β-Methyl-β-oxy-Δγ,δ-hexen-säure-methylester wurde mit wasserfreiem Natriumbisulfat (⅓ des Gewichts vom Oxyester) 20 Minuten lang auf etwa 80° erhitzt und anschließend der Destillation im Vakuum unterworfen. Ausbeute 50 % d. Th. Die Verseifung des Esters wurde mit 1½ Molen 10%iger alkoholischer Kalilauge durchgeführt.

d) Methyl-(2)-hexadien-(2,4)-säure-(1)-amid. Die Säure wurde in der üblichen Weise mit Thionylchlorid behandelt, nach dem Abkühlen in Aceton-Kohlensäureschnee mit flüssigem Ammoniak versetzt, das abgeschiedene Amid hierauf mit heißem Aceton behandelt und vom ungelösten Ammoniumchlorid abfiltriert. Das Filtrat wurde mit Tierkohle gekocht, filtriert und zum Krystallisieren gestellt. Schmelzp. 136—141°.

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17 [1]) Chem. Z. 1928, I, 2207.
18 [2]) Ber. chem. Ges. 65, 655 (1932).

8. Octatrien-(2,4,6)-säure-(1) (I, n = 3). Aus mit Ammoniak gereinigtem Hexadienal nach R. Kuhn und M. Hoffer[1]). Das Hexadienal stellten wir nach der von R. Kuhn, W. Badstübner und Ch. Grundmann[2]) angegebenen Methode dar. Die einmal aus verdünntem Methanol umkrystallisierte Säure schmolz bei 184,5° (k. Th.).

9. Dekatetraen-(2,4,6,8)-säure-(1) (I, n = 4). Nach R. Kuhn und M. Hoffer[3]). Das Octatrienal gewannen wir unter Verwendung von Piperidin-Eisessig als Katalysator. Die einmal aus Eisessig umkrystallisierte Säure schmolz bei 222° (k. Th.).

10. Octatrien-(2,4,6,)-säure-(1)-amid (XI). 30 g Triensäure wurden mit 28 g frisch destilliertem Thionylchlorid versetzt und 30 Minuten am Dampfbad unter Rückfluß (Chlorcalcium-Rohr) erhitzt. Hierauf dampften wir im Vakuum das überschüssige Thionylchlorid ab, fügten 150 ccm trockenen Äther zu, kühlten in einem Aceton-Kohlensäureschnee-Gemisch gut ab und versetzten die ätherische Lösung vorsichtig mit flüssigem Ammoniak. Nach dem Verdampfen des Äthers und des überschüssigen Ammoniaks wurde der Rückstand mit Wasser ausgelaugt, das Säure-amid abfiltriert und aus kochendem Alkohol umkrystallisiert. Ausbeute 20,2 g; aus der Mutterlauge gewannen wir weitere 5,9 g. Die Substanz krystallisiert aus heißem Wasser in weißen, gefiederten flachen Nadeln, die ohne Zersetzung bei 208—209° schmelzen.

4,020, 4,122 mg Subst.: 10,34, 10,60 mg CO_2, 2,95, 3,02 mg H_2O. — 4,261, 4,227 mg Subst.: 0,381 (21°, 741 mm), 0,383 (21°, 743 mm) ccm N.

$C_8H_{11}ON$ (137,1)	Ber.	C 70,02	H 8,09	N 10,21
	Gef.	„ 70,15, 70,13	„ 8,21, 8,19	„ 10,12, 10,28.

11. Dekatetraen-(2,4,6,8)-säure-(1)-amid (XV). 15 g Tetraensäure vom Schmelzp. 224° (k. Th.) wurden fein gepulvert und nach Zusatz von 40 ccm Benzol (über Natrium getrocknet) mit 16 g frisch destilliertem Thionylchlorid versetzt. Nach 35 Minuten Erwärmen am Dampfbad wurde durch Absaugen im Vakuum vom überschüssigen Thionylchlorid befreit und von der nicht in Reaktion getretenen Tetraensäure (2,5 g) abfiltriert. Das Filtrat wurde auf −15° abgekühlt und mit einem Überschuß an flüssigem Ammoniak versetzt, worauf nach erfolgter Umsetzung sofort vom ausgefallenen Niederschlag abfiltriert wurde. Denselben behandelten wir zwecks Entfernung des Ammoniumchlorids mit kaltem Wasser und krystallisierten hierauf den Rückstand aus absolutem Alkohol um. Ausbeute 9,6 g. Die Substanz krystallisiert aus Alkohol in orange gefärbten Prismen, die ohne Zersetzung bei 227° (k. Th.) schmelzen.

4,174 mg Subst.: 11,18 mg CO_2, 2,99 mg H_2O. — 3,854 mg Subst.: 0,282 ccm N (20°, 750 mm).

$C_{10}H_{13}ON$ (163,1)	Ber.	C 73,57	H 8,03	N 8,57
	Gef.	„ 73,38	„ 8,05	„ 8,41.

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19 [1]) Ber. chem. Ges. 64, 1978 (1931).
20 [2]) Ber. chem. Ges. 69, 98 (1936).
21 [3]) Ber. chem. Ges. 63, 2164 (1930).

Lösungsmittels wurde der Rückstand im Vakuum destilliert, wobei 24,5 g (21% d. Th.) unter 11 mm bei 74,5—78° übergingen. Nach abermaliger Destillation siedete das 5-Methyl-furfurol unter 12 mm bei 75,5°. $n_D^{18°}$ = 1,5248. Es läßt sich unter Vakuum im Dunkeln längere Zeit unzersetzt aufbewahren.

17. 5-Methyl-brenzschleimsäure-amid (VI). — a) 5-Methyl-brenzschleimsäure. 30 g 5-Methyl-furfurol wurden mit 50 ccm 96%igem Alkohol und einer Lösung von 92,6 g Silbernitrat in 100 ccm Wasser versetzt, wozu unter ständigem Rühren eine Lösung von 32,7 g Natriumhydroxyd in 100 ccm Wasser zutropfen gelassen wurde. Nach Beendigung der Oxydation filtrierten wir das ausgeschiedene Silber ab, dampften das Filtrat bis fast zur Trockne ein, säuerten an und stellten die wäßrige Lösung in den Eisschrank, nachdem sie mit Tierkohle entfärbt worden war. Die 5-Methyl-brenzschleimsäure krystallisiert in großen Rhomben und schmilzt bei 109—110° (k. Th.). Ausbeute an schmelzpunktreiner Substanz 76—91% d. Th.

b) 5-Methyl-brenzschleimsäure-amid. 31 g 5-Methyl-brenzschleimsäure wurden mit 45 g Thionylchlorid und 20 ccm trockenem Äther 30 Minuten am Dampfbad erhitzt. Nach dem Abkühlen trugen wir die ätherische Lösung des Säurechlorids langsam in mit Ammoniak gesättigten Äther ein, wobei sich die Bildung des Amids ruhig vollzog. Nach dem Verdunsten des Äthers wurde die heiße wäßrige Lösung des Rückstandes mit Tierkohle entfärbt und in den Eisschrank gestellt. Ausbeute an schmelzpunktsreiner Substanz = 25 g (81% d. Th.). Schmelzp. 132 bis 138° (k. Th.).

18. β-[5-Methyl-furyl-(2)]-acrylsäure-amid (VIII). — a) β-[5-Methyl-furyl-(2)]-acrylsäure[1]. 28 g 5-Methyl-furfurol wurden mit 27 g Malonsäure und 28 g Pyridin zunächst über Nacht bei gewöhnlicher Temperatur stehen gelassen, hierauf am Dampfbad unter Rückfluß erhitzt, bis keine Kohlensäure mehr entwickelt wurde (etwa 3 Stunden). Dann kühlten wir das Reaktionsprodukt mit Eis und säuerten mit verdünnter Schwefelsäure an, wobei ein brauner Niederschlag ausfiel, der in 2 n-Natronlauge gelöst und mit Tierkohle in der Hitze entfärbt wurde. Die durch verdünnte Salzsäure wieder ausgefällte β-[5-Methyl-furyl-(2)]-acrylsäure krystallisierten wir aus sehr verdünntem Methanol um, wobei schneeweiße, lange Nadeln vom Schmelzp. 157° (k. Th.) erhalten wurden. Ausbeute 28 g (70% d. Th.).

b) β-[5-Methyl-furyl-(2)]-acrylsäure-amid. 40 g β-[5-Methyl-furyl-(2)]-acrylsäure wurden mit 39 g frisch destilliertem Thionylchlorid und 100 ccm trockenem Äther 90 Minuten am Dampfbad erhitzt und hierauf im Vakuum destilliert. Ausbeute 44,5 g (99,5% d. Th.). Das β-[5-Methyl-furyl-(2)]-acrylsäure-chlorid ist eine schwach gelb gefärbte, stark lichtbrechende, nicht unangenehm riechende Flüssigkeit, die

[1]) Herrn cand. chem. H. Ruelius, der vergleichende Versuche über die Synthese dieser Säure ausgeführt hat, danken wir für seine Unterstützung bestens. Die Perkinsche Synthese mit 5-Methyl-furfurol gibt sowohl bei Anwendung von Kaliumacetat (24% d. Th.) als auch von Natriumacetat (15% d. Th.) erheblich schlechtere Ausbeuten als die oben beschriebene Synthese nach Doebner (70% d. Th.).

unter 9 mm bei 124° siedet und zu lichtbrechenden, wohl ausgebildeten Täfelchen erstarrt, welche bei 37° schmelzen.

Das Säurechlorid wird in Äther gelöst, mit Aceton-Kohlensäureschnee gut gekühlt und mit einem geringen Überschuß von flüssigem Ammoniak versetzt. Man dampft den Äther ab und behandelt den Rückstand mit warmem Aceton, filtriert vom Ammoniumchlorid ab und läßt das Filtrat erkalten. Das β-[5-Methyl-furyl-(2)]-acrylsäure-amid krystallisiert aus verdünntem Aceton in sternförmig angeordneten Prismen, aus absolutem Aceton in derben Drusen. Ausbeute 38,2 g (98% d. Th.). Schmelzp. 130—131° (k. Th.). Löslich in Wasser, Alkohol, Methanol, Aceton.

4,023, 4,187 mg Subst.: 9,340, 9,765 mg CO_2, 2,190, 2,260 mg H_2O. — 4,288, 8,995 mg Subst.: 0,337, 0,325 ccm N (23°, 752, 749 mm).

$C_8H_9O_2N$ (151)	Ber.	C 63,57	H 5,96	N 9,27
	Gef.	„ 63,32, 63,60	„ 6,09, 6,04	„ 8,97, 9,25.

II. Fütterungsversuche.

Gesunde, gut genährte Kaninchen wurden mit der Schlundsonde gefüttert, wobei die täglich gegebene Menge von der Art der Substanz abhängig gemacht wurde.

1. Sorbinsäure wurde in der genau berechneten Menge 2n-Natronlauge gelöst. Das Kaninchen erhielt täglich 2mal 5 g. Pharmakologische Wirkungen waren bei dieser Menge nicht zu beobachten und die Tiere überstanden den Fütterungsversuch ausgezeichnet. Gewicht der Tiere: 3320 bzw. 3380 g.

2. Octatrien- und Dekatetraensäure wurden staubfein zerrieben und in Wasser suspendiert mit der Schlundsonde gegeben, 2mal 1—2 g je Tag und Tier. Die Tiere (Gewicht 3220 bzw. 3320 g) zeigten keinerlei auffallende Erscheinungen.

3. Sorbinsäure-methylester und -äthylester wurden unverdünnt gegeben, 2mal 5 g je Tag und Tier. Bei der Fütterung von Sorbinsäure-methylester fiel der außerordentliche Heißhunger des Kaninchens (Gewicht 3220 g) auf und die während dieses Versuches anfallende große Menge Harn.

4. Sorbinsäure-amid. Das fein zerriebene und in Wasser suspendierte Amid wurde in steigenden Mengen, 1—4 g je Fütterung, gegeben. Die bei einer Dosis von 4 g auftretende pharmakologische Wirkung äußert sich in einer Lähmung der Gliedmaßen, beginnend bei den hinteren Extremitäten, Bewußtlosigkeit und in einer gesteigerten Tätigkeit des Herzens. Füttert man von Anfang an Mengen von 3 g, so tritt bald eine außerordentliche Verzögerung der Atmung und Exitus ein. Bei langsamer Gewöhnung überstehen die Tiere den Versuch ohne

jeden Schaden, selbst wenn von Zeit zu Zeit vorübergehende Lähmungen eintreten. Gewicht des Tieres: 3320 g.

5. **Sorbinsäure-anilid** wurde fein gepulvert, in Wasser aufgeschlämmt und in Mengen von 2—6 g je Tag und Tier gegeben. Es konnte, auch in Fütterungsversuchen über lange Dauer, keine besondere Wirkung des Anilids auf den Organismus beobachtet werden.

6. **Sorbinsäure-methyl-amid.** Die Substanz wurde gepulvert, mit Wasser angeteigt und in steigenden Mengen von 0,5—2,0 g auf einmal gegeben. Wie gewöhnlich wurde 2mal im Tag gefüttert. Bei einer Menge von 2,5—3,0 g je Fütterung waren nach ungefähr 5 Minuten die hinteren Gliedmaßen gelähmt, der Herzschlag intensiv, die Atmungsfrequenz sehr rasch und das Atemvolumen klein. Nach 25 Minuten war totale Lähmung und Bewußtlosigkeit eingetreten, wobei das Herz sehr ruhig schlug und der Atmungszustand der gleiche blieb. Ende der Wirkung nach ungefähr 2 Stunden.

7. **β-Methyl-sorbinsäure-amid.** Das nicht ganz reine Präparat (Schmelzp. 136—141°) wurde in Wasser suspendiert in zwei Dosen zu 0,5 und 2,0 g gegeben (innerhalb von 17 Stunden), worauf nach kurzer Zeit von den rückwärtigen Gliedmaßen her Lähmungserscheinungen einsetzten, die sich mit heftiger Wirkung über den ganzen Körper ausbreiteten. Herzschlag unregelmäßig, Atmung außerordentlich verlangsamt, Atemtiefe sehr klein. Beginnendes Ende der Lähmung und der Bewußtlosigkeit nach 2 Stunden.

8. **Crotonsäure-amid.** Die besondere Giftigkeit dieser Substanz gestattete es nicht, mehr als 0,5—1,0 g je Tag zu geben. Dennoch trat nach Fütterung einer Gesamtmenge von 7,5 g Exitus ein. Gewicht des Tieres: 3180 g. Füttert man 2mal 1,5 g, so tritt nach ungefähr 12 Stunden Exitus ein. (Gewicht des Tieres: 3430 g.) In einem anderen Versuch verendete das Kaninchen bereits nach 2 Stunden, bei Gaben von 2,0—3,0 g innerhalb 7 Stunden (Gewicht des Tieres: 3320 g). Es treten auch hier wieder Lähmungserscheinungen auf, die zu charakteristischen krampfartigen Streckstellungen der Gliedmaßen und des Kopfes führen. Bei Fütterungsversuchen über eine längere Zeit, treten nekrotische Veränderungen der Nieren auf.

9. **Crotonsäure-anilid.** Das Anilid wurde fein gepulvert, in Wasser suspendiert, in Mengen von 2mal 2 g je Tag gefüttert. Ein männliches Tier vertrug die Fütterung ohne jede besondere

Erscheinung, während ein weibliches Kaninchen nach der ersten Gabe von 2 g eine heftige Lähmung der Gliedmaßen bekam, von denen es sich nach der Unterbrechung des Fütterungsversuches rasch und vollständig erholte.

10. **β,β-Dimethyl-acrylsäure-amid** (III) wurde fein gepulvert, in Wasser suspendiert, in steigenden Mengen von 2mal 0,5—2 g je Tag gefüttert. Der Versuch wurde vom Tier gut überstanden, ohne jede besondere Erscheinung. Gewicht des Tieres: 4200 g.

11. **β-Methyl-β-äthyl-acrylsäure-amid** wurde wie gewöhnlich in fester Form gegeben, 2mal 2 g je Tag, wobei jedesmal eine Lähmung der hinteren Gliedmaßen zu beobachten war, die ohne weitere Folgeerscheinung blieb. Gewicht des Tieres: 2930 g.

12. **5-Methyl-brenzschleimsäure-amid** (VI). Die in Wasser aufgeschlämmte Substanz wurde in Dosen von 2mal 1 g bis 2mal 2 g je Tag gefüttert, wobei keinerlei pharmakologische Wirkungen zu beobachten waren. Gewicht des Tieres: 3320 g.

13. **β-[5-Methyl-furyl-(2)]-acrylsäure-amid** (VIII). Wir fütterten das in Wasser suspendierte Amid zunächst in Mengen von 2mal 0,5 g je Tag, hierauf gaben wir 1,0 g, wobei nach 10 Minuten Lähmung der Gliedmaßen, von den rückwärtigen beginnend, eintrat, die nach etwa 1 Stunde aufgehoben war. Bei einer langsamen Steigerung der Dosis traten die Lähmungserscheinungen immer schwächer auf, bis sie ganz verschwanden und auch bei einer Menge von 3,0 g nicht mehr zu beobachten waren. Gewicht des Tieres: 4940 g.

14. **Octatriensäure-amid** (XI). Gefüttert, wie vorhergehend beschrieben, in Mengen von 2mal 1,0 g bis 2mal 2,0 g je Tag. Nach einer Gesamtmenge von 14,0 g exitus. Gewicht des Tieres: 2890 g. Leber körnig (weiß) durchsetzt, Magenwand zeigt zahlreiche Blutknöllchen, Mageninhalt ist dunkelrotbraun gefärbt.

15. **Dekatetraensäure-amid** (XV). Das fein gepulverte Amid wurde mit Wasser verrührt und in Mengen von 2mal 1 g je Tag gegeben. Die Substanz übte in dieser Konzentration keine sichtbaren Wirkungen auf den Organismus aus. Gewicht des Tieres: 2930 g.

III. Isolierung und Identifizierung der Dicarbonsäuren bzw. Dicarbon-amidsäuren aus dem Harn.

Die Isolierung der Oxydationsprodukte gestaltete sich insofern einfach, als dieselben fast durchweg in Wasser schwerlösliche Körper darstellen.

Der unter Toluol gesammelte, alkalisch reagierende Harn wurde nach der letzten Fütterung noch weitere 2 Tage aufgefangen, hierauf von den Sedimenten abfiltriert und im Vakuum (15 mm) auf 100—200 ccm eingedampft. Nach dem Abkühlen wurde durch Zentrifugieren von den abgeschiedenen Salzen befreit und die klare Flüssigkeit mit 2n-Salzsäure angesäuert (Kongo). Wir ließen dann einige Stunden im Eisschrank stehen, filtrierten die in vielen Fällen bereits krystallin vorliegende Dicarbonsäure bzw. deren Derivat, ab und reinigten dieselbe durch Behandlung des Ammoniumsalzes (in 2n-Ammoniak gelöst) mit Adsorptionsmitteln, wie Carbo medicinalis „Merck", Frankonit KL, Floridin XS und Floridin XXF. Es ist dabei größte Vorsicht zu üben, da besonders bei Verwendung von Carbo medicinalis „Merck" die Dicarbon-amidsäuren und die Mucon-anilidsäure eine vollständige Zersetzung erleiden können. In manchen Fällen genügt ein längeres Kochen mit Wasser in Gegenwart der genannten Entfärbungsmittel, um die stark ungesättigten Dicarbonamidsäuren in nicht mehr krystallisierende Produkte zu verwandeln.

1. 15 g **Sorbinsäure** gaben 0,200 g **Muconsäure** (Rohprodukt, Schmelzp. 250—260°), die zur Identifizierung in den **Dimethylester** übergeführt wurde[1]). Ausbeute an reinem Ester: 38 mg.

3,810, 3,802 mg Subst.: 5,10, 5,08 ccm n/100-NaOH.

$C_6H_6O_4$ (142) Ber. Äquiv.-Gew. 71 Gef. Äquiv.-Gew. 74, 75.

In einem zweiten Versuch erhielten wir aus 30 g Sorbinsäure 220 mg schmelzpunktreine Muconsäure, aus der wir 90 mg reinen Dimethylester gewannen. Die bei der Herstellung des Esters anfallende alkoholische Mutterlauge roch intensiv nach Sorbinsäure-methylester!

Schmelzpunkt der aus dem Harn isolierten Muconsäure: 299—303° (k. Th.). Schmelzpunkt des Muconsäure-dimethylester: 157°; Misch-Schmelzpunkt mit einem synthetischen Präparat: 156°. Ausbeute an Dicarbonsäure aus dem Harn (berechnet auf den gewonnenen Dimethylester): 0,17 bzw. 0,19% d. Th.

2. Nach Fütterung von 22 g **Sorbinsäure-methylester** konnten wir aus dem Harn **keine Muconsäure** isolieren.

3. 30 g **Sorbinsäure-äthylester** lieferten 145 mg **Muconsäure** [0,5% d. Th. vom Schmelzp. 300° (k. Th.)].

3,401, 3,420 mg Subst.: 4,76, 4,80 ccm n/100-NaOH.

$C_6H_6O_4$ (142) Ber. Äquiv.-Gew. 71 Gef. Äquiv.-Gew. 71,4, 71,2.

4. Aus 7,6 g **Sorbinsäure-amid** erhielten wir 3,1 g **Mucon-amidsäure** (32,2% d. Th.). Die Mucon-amidsäure krystallisiert aus Wasser in

[1]) R. Kuhn u. Ch. Grundmann, Ber. chem. Ges. 69, 1757 (1936).

farblosen, glänzenden Nädelchen, die bei 276—277° = 281—282° (k. Th.) unter Zersetzung schmelzen. 100 ccm Wasser lösen bei 4° 0,0129 g Mucon-amidsäure. Schwer löslich in heißem Eisessig, unlöslich in Alkohol.

6,206 mg Subst.: 4,39 ccm n/100-NaOH. — 1,500 mg Subst. in 1,0 ccm n/10-Na_2CO_3 mit 8,3 mg Platinoxyd nach 20 Minuten Endwert. — 4,577 mg Subst.: 0,385 ccm N_2 (23°, 751 mm). — 4,183 mg Subst.: 7,820 mg CO_2, 1,835 mg H_2O.

	$C_6H_7O_3N$ (141)				
Ber.	Äquiv.-Gew. 141	⌈ 2,00	C 51,06	H 4,96	N 9,92
Gef.	Äquiv.-Gew. 141	⌈ 2,03	„ 50,98	„ 4,91	„ 9,59.

Mucon-amidsäure reagiert mit PCl_5 viel langsamer als Muconsäure und gibt nach Zusatz von absolutem Methanol *trans-trans*-**Muconsäure-dimethylester**, der bei 157—157,5° (k. Th.) schmilzt. PCl_5 verändert die Säureamid-Gruppe zum Chlorimid, das bei der Einwirkung von Methanol über die Methoxy-imid-Verbindung den Ester gibt.

4,060, 4,227 mg Subst.: 8,400, 8,780 mg CO_2, 2,215, 2,340 mg H_2O.

$C_8H_{10}O_4$ (170,1)	Ber.	C 56,43	H 5,93
	Gef.	„ 56,43, 56,65	„ 6,10, 6,19.

Verseifung der Mucon-amidsäure. 100 mg Mucon-amidsäure wurden mit 5 ccm 30%iger alkoholischer Kalilauge und 10 ccm Wasser 90 Minuten am Dampfbad erhitzt, nach dem Erkalten mit 2 n-Salzsäure angesäuert und filtriert. Den Niederschlag lösten wir hierauf in 2n-Natronlauge, behandelten in der Hitze mit Tierkohle, filtrierten ab und säuerten das Filtrat an, wobei die Muconsäure in schneeweißen Kryställchen ausfiel. Nach einigem Stehen in der Kälte wurde abgesaugt, gut mit Wasser gewaschen und über Phosphorpentoxyd getrocknet. Schmelzp. 299° (k. Th.).

5,961, 5,295 mg Subst.: 8,45, 7,40 ccm n/100-NaOH.

$C_6H_6O_4$ (142) Ber. Äquiv.-Gew. 71 Gef. Äquiv.-Gew. 70,5, 71,5.

5. Aus 19,5 g **Sorbinsäure-methyl-amid** erhielten wir 9,2 g (43,8% d. Th.) **Mucon-methylamid-säure** vom Schmelzp. 217° (k. Th.) unter Zersetzung. Mucon-methylamid-säure löst sich in heißem Wasser, in viel heißem Alkohol, in heißem Methanol, in Pyridin und ist unlöslich in Äther, Chloroform und schwer löslich in Essigester.

4,186, 4,171 mg Subst.: 8,335, 8,33 mg CO_2, 2,205, 2,155 mg H_2O. — 5,600, 5,396 mg Subst.: 3,61, 3,47 ccm n/100-NaOH. — 4,001, 3,945 mg Subst.: 0,321 (23°, 754 mm), 0,311 (22°, 755 mm) ccm N_2.

$C_7H_9O_3N$ (155)	Ber.	Äquiv.-Gew. 155	C 54,19	H 5,80	N 9,03
	Gef.	„ 155	„ 54,31	„ 5,90	„ 9,18
	„	„ 155	„ 54,46	„ 5,78	„ 9,06.

6. 20 g **Sorbinsäure-anilid** gaben 2,7 g **Mucon-anilid-säure** (11,5% d. Th.); in einem zweiten Versuch erhielten wir aus 18 g Sorbinsäure-anilid 7,5 g Mucon-anilid-säure (36% d. Th.) und in einem dritten Versuch isolierten wir nach der Verfütterung von 17 g Sorbinsäure-anilid

aus dem Harn 3,1 g Mucon-anilid-säure (15,7 % d. Th.). Die Mucon-anilid-säure wird aus alkalischer Lösung mit verdünnter Salzsäure stets amorph gefällt; der Niederschlag geht aber beim Erhitzen der wäßrigen Suspension in den krystallinischen Zustand über. 3000 Teile kochendes Wasser lösen 1 Teil Mucon-anilid-säure; sie ist gegen kochende konzentrierte Salzsäure beständig, worin sie sich löst und woraus sie beim Erkalten wieder ausfällt. Aus heißem Wasser krystallisiert die Substanz in unregelmäßigen Plättchen, die bei 261—263° (k. Th.) unter Zersetzung, Braunfärbung und ohne zu sublimieren schmelzen. Die amorphe Mucon-anilid-säure hingegen sintert (nach dem Trocknen im Vakuum über Phosphorpentoxyd) bei 250°, zersetzt sich bei 310° und gibt ein Sublimat von goldgelben Nädelchen. Mucon-anilid-säure ist schwer löslich in Wasser, Benzol, löslich in Essigester, gut löslich in absolutem Alkohol, leicht löslich in Methanol, unlöslich in Äther und Chloroform.

3,945 mg Subst.: 9,545 mg CO_2, 1,85 mg H_2O. — 8,065 mg Subst.: 3,70 ccm n/100-NaOH. — 3,656 mg Subst.: 0,212 ccm N (20°, 741 mm).

$C_{12}H_{11}O_2N$ (217,1)	Ber.	Äquiv.-Gew. 217,1	C 66,32	H 5,11	N 6,45
	Gef.	„ 217	„ 65,99	„ 5,24	„ 6,59.

7. 2,5 g β-Methyl-sorbinsäure-amid gaben nach dem Verfüttern 1,91 g (62 % d. Th.) β-Methyl-mucon-amidsäure. Schmelz. 259—261° (k. Th.). 1000 Teile kochendes Wasser lösen 4,1 Teile β-Methyl-mucon-amidsäure. Sie krystallisiert aus Wasser in langen, feinen, farblosen Prismen. Die neue Substanz ist löslich in heißem Methanol, schwer löslich in heißem Alkohol und in Wasser, kaum löslich in Essigester und in Chloroform.

3,963, 4,134 mg Subst.: 7,865, 8,180 mg CO_2, 2,080, 2,145 mg H_2O. — 5,238, 5,300 mg Subst.: 3,37, 3,42 ccm n/100-NaOH. — 3,894, 3,870 mg Subst.: 0,312 (22°, 744 mm), 0,311 (21°, 742 mm) ccm N.

$C_7H_9O_2N$ (155)

Ber.	Äquiv.-Gew. 155	C 54,19	H 5,80	N 9,03
Gef.	„ 155, 155	„ 54,13, 53,92	„ 5,87, 5,80	„ 9,08, 9,11.

Verseifung der β-Methyl-mucon-amidsäure. 100 mg β-Methyl-mucon-amidsäure wurden mit 5 ccm 30 %iger wäßriger Kalilauge 60 Minuten am Dampfbad erhitzt, nach dem Erkalten mit 2n-Salzsäure angesäuert, wobei ein schneeweißer Niederschlag ausfiel, der abfiltriert, getrocknet und gewogen wurde. Ausbeute 79,6 mg (79,6 % d. Th.). Aus Wasser umkrystallisiert. Schmelzpunkt der so erhaltenen β-Methyl-muconsäure 231—232° (k. Th.); Mischschmelzpunkt mit einem synthetischen Präparat[1]) 231 bis 232° (k. Th.).

4,190, 4,128 mg Subst.: 5,40, 5,37 ccm n/100-NaOH.

$C_7H_8O_4$ (156) Ber. Äquiv.-Gew. 78 Gef. Äquiv.-Gew. 77,5, 78,7.

8. Nach der Verfütterung von 6,3 g Octatrien-säure bzw. 8,0 g Dekatetraen-säure konnten wir aus dem Harn *keine* Dicarbonsäure isolieren.

9. 14,0 g Octatriensäure-amid gaben 7,123 g (42 % d. Th.) Hexatrien-(2,4,6)-dicarbon-(1,6)-amidsäure-(1). Beim Fällen der Substanz

[1]) R. Kuhn u. Ch. Grundmann, Ber. chem. Ges. 69, 1757 (1936).

aus alkalischer Lösung mit verdünnter Salzsäure erhält man feine, sternförmige Nädelchen. Die Hexatrien-dicarbonamidsäure krystallisiert aus viel kochendem Wasser in farblosen unregelmäßigen hexagonalen Plättchen, die sich (bei 190° in den Block gebracht) bei 263° (k. Th.) zersetzen, ohne zu schmelzen. Hexatrien-dicarbon-amidsäure (XII) ist löslich in heißem Wasser und verdünntem Alkali, unlöslich in Essigester, Alkohol, Chloroform, Schwefelkohlenstoff und konz. Salzsäure.

4,054 mg Subst.: 8,53 mg CO_2, 2,00 mg H_2O. — 6,404 mg Subst.: 3,77 ccm n/100-NaOH. — 4,272 mg Subst.: 0,310 ccm N (19°, 742 mm).

$C_8H_9O_2N$ (167)	Ber.	Äquiv.-Gew. 167	C 57,48	H 5,38	N 8,37
	Gef.	„ 170	„ 57,38	„ 5,52	„ 8,28.

Verseifung der Hexatrien-(2,4,6)-dicarbon-(1,6)-amidsäure-(1). 1,0 g Amidsäure (XII) wurden mit 10 ccm 30 %iger wäßriger Kalilauge versetzt und 2 Stunden am Dampfbad erhitzt. Nach dem Abkühlen wurde mit Salzsäure angesäuert, der ausgefallene Niederschlag abfiltriert und getrocknet. Ausbeute: 0,60 g (60 % d. Th.). Die scharf getrocknete Hexatrien-1,6-dicarbonsäure (XIII) wurde mit Phosphorpentachlorid und Methanol wie üblich in den Dimethylester übergeführt. Ausbeute: 0,640 g (99 % d. Th.). Aus Methanol umkrystallisiert. Schmelzpunkt des so erhaltenen Hexatrien-1,6-dicarbonsäure-dimethylesters 169,5—170,5° (k. Th.); Mischschmelzpunkt mit einem synthetischen Vergleichspräparat[1]) 169,5—171° (k. Th.).

4,128, 4,013 mg Subst.: 9,245, 8,97 mg CO_2, 2,28, 2,20 mg H_2O.

$C_{10}H_{12}O_4$ (196,1)	Ber.	C 61,19	H 6,17
	Gef.	„ 61,08, 60,96	„ 6,18, 6,13.

10. Aus 7,0 g Dekatetraensäure-amid erhielten wir 1,7 g (20 % d. Th.) Octatetraen-(2,4,6,8)-dicarbon-(1,8)-amidsäure-(1), welche aus Wasser körnige, gelbe Krystalle bildete. Die alkalische Lösung der Octatetraen-dicarbon-amidsäure (XVI) fluoresciert vor der Analysenlampe bläulichgrün. Das nicht ganz reine Präparat schmolz unter Zersetzung bei 258° (k. Th.).

4,945 mg Subst.: 0,311 ccm N (24°, 751 mm).

$C_{10}H_{11}O_2N$ (193,1) Ber. N 7,25 Gef. N 7,12.

Verseifung der Octatetraen-(2,4,6,8)-dicarbon-(1,8)-amidsäure-(1). 800 mg Amidsäure (XVI) wurden mit 10 ccm 30 %iger wäßriger Kalilauge versetzt und 2½ Stunden am Dampfbad erhitzt. Nach dem Abkühlen wurde mit Salzsäure angesäuert, vom Niederschlag abfiltriert und scharf getrocknet. Ausbeute 0,440 g. Die trockene Octatetraen-1,8-dicarbon-säure wurde mit 0,8 g Phosphortrichlorid und Methanol wie üblich in den Dimethylester übergeführt. Ausbeute 0,240 g. Aus Eisessig umkrystallisiert schmolz der Dimethylester bei 212° (k. Th.); Mischschmelzpunkt mit einem synthetischen Vergleichspräparat[1]) 213° (k. Th.).

3,884 mg Subst.: 9,270 mg CO_2, 2,200 mg H_2O.

$C_{12}H_{14}O_4$ (222,1) Ber. C 64,84 H 6,35 Gef. C 65,09 H 6,34.

[1]) R. Kuhn u. Ch. Grundmann, a. a. O.

11. Wegen der bedeutenden Giftigkeit des **Crotonsäureamids** konnten wir nur geringe Mengen füttern. Es gelang uns nicht, aus dem Harn Oxydationsprodukte in reiner Form zu isolieren. Die bei der Aufarbeitung des Harns spurenweise anfallenden krystallinischen Abbauprodukte genügten nicht für eine genaue Untersuchung.

12. 21 g **Crotonsäure-anilid** gaben nach dem Verfüttern 3,2 g (14% d. Th.) schmelzpunktreines **N-Crotonyl-p-aminophenol**. Der beim Eindampfen des alkalischen, stark dunkel gefärbten Harns ausfallende Niederschlag wurde abzentrifugiert und nach dem Trocknen aus Aceton umkrystallisiert. Entfärbt man die acetonische Lösung mit Tierkohle, so krystallisiert daraus der neue Körper in derben, schneeweißen Prismen, die sich in Aceton, Alkohol, heißem Wasser, kochendem Essigester, Eisessig, Methyläthyl-keton und in Pyridin lösen und die in Äther, Petroläther, Chloroform und Benzol unlöslich sind. Aus den Aceton-Mutterlaugen gewannen wir nach dem Einengen außerdem 150 mg reinen Harnstoff. 1000 Teile Wasser lösen bei 20° 400 mg N-Crotonyl-p-aminophenol, welches bei 189,5—190,5° (k. Th.) schmilzt. Das N-Crotonyl-p-aminophenol gibt in verdünntem Methanol gelöst, eine schöne grüne Eisentrichloridreaktion. Mischschmelzpunkt mit dem synthetischen Präparat 189,5—190,5°.

1,690 mg Subst. in 20 ccm Eisessig mit 10,3 mg Palladiumoxydul nach 8 Minuten Endwert.

4,207, 3,890 mg Subst.: 10,435, 9,65 mg CO_2, 2,37, 2,225 mg H_2O.

$C_{10}H_{11}O_2N$ (177,1)	Ber.	f 1,00	C 67,76	H 6,27
	Gef.	f 1,06	„ 67,61, 67,65	„ 6,30, 6,40.

Zur Überführung in das **N-Butyryl-p-aminophenol** wurden 800 mg **N-Crotonyl-p-aminophenol** in 70 ccm Essigester und 20 ccm Alkohol gelöst, mit 500 mg **Palladiumoxydul**[1]) versetzt und bis zum Stillstand der Wasserstoffaufnahme hydriert. Nach dem Abfiltrieren des Palladiums dampften wir das Lösungsmittel im Vakuum ab und krystallisierten den Rückstand aus Aceton um. $FeCl_3$-Reaktion in wäßrigem Methanol grün. Das N-Butyryl-p-aminophenol krystallisierte aus Aceton in regelmäßigen, 6eckigen, glasklaren Plättchen, aus Wasser in dichtgefiederten Nadeln. Schmelzp. 138 bis 139° (k. Th.), Mischschmelzpunkt mit dem synthetischen Präparat 138 bis 139° (k. Th.). Die Substanz ist leicht löslich in Aceton, Essigester, heißem Wasser und Methanol, löslich in Äther, unlöslich in Petroläther.

[1]) R. L. Shriner u. R. Adams, J. Amer. Chem. Soc. 46, 1683 (1924).

3,915, 4,004 mg Subst.: 9,61, 9,86 mg CO_2, 2,525, 2,58 mg H_2O.

$C_{10}H_{13}O_2N$ (179,1)	Ber.	C 67,00	H 7,32
	Gef.	„ 66,94, 67,16	„ 7,21, 7,21.

13. 18 g **β,β-Dimethyl-acrylsäure-amid** (III) lieferten 10,1 g (43% d. Th.) **Mesacon-α-amidsäure**, die bereits von R. Anschütz[1]) beschrieben wurde. Sie ist löslich in heißem Wasser, in heißem Alkohol, unlöslich in kaltem Wasser und Äther. Aus heißem Wasser krystallisiert die Mesacon-α-amidsäure in farblosen, derben Krystallen, die bei 223° (k. Th.) schmelzen. R. Anschütz gibt 222° an.

4,137 mg Subst.: 7,100 mg CO_2, 2,065 mg H_2O. — 5,750, 5,458 mg Subst.: 4,42, 4,17 ccm n/100-NaOH. — 4,140 mg Subst.: 0,386 ccm N (21°, 751 mm).

$C_5H_7O_3N$ (129)	Ber.	Äquiv.-Gew. 129	C 46,51	H 5,42	N 10,85
	Gef.	„ 130, 130	„ 46,80	„ 5,59	„ 10,70.

14. Nach dem Füttern von 13 g **5-Methyl-brenzschleimsäure-amid** (VI) isolierten wir 5,1 g (32% d. Th.) **Furan-dicarbon-(2,5)-amidsäure-(2)**. Diese Substanz ist von I. Phelps und W. Hale[2]) beschrieben worden, vom Schmelzp. 280—281° (korr.). Sie ist löslich in heißem Wasser, unlöslich in Alkohol und schwer löslich in Eisessig. Wir isolierten die Furan-dicarbon-(2,5)-amidsäure-(2) als dimorphen Körper, der aus Eisessig in prachtvollen, langen, seidenglänzenden Nadeln krystallisiert und aus Wasser in Form von gedrungenen Prismen und in Form von langen, feinen Nadeln abgeschieden wird. Das Krystallisationsbestreben der Prismen ist größer, als das der Nadeln. Trennung durch fraktionierte Krystallisation. Schmelzpunkt der Prismen 283° (k. Th.).

4,125 mg Subst.: 7,025 mg CO_2, 1,195 mg H_2O. — 7,002, 8,549 mg Subst.: 4,50, 5,57 ccm n/100-NaOH. — 4,535 mg Subst.: 0,359 ccm N (23°, 751 mm).

$C_6H_5O_4N$ (155)	Ber.	Äquiv.-Gew. 155	C 46,45	H 3,22	N 9,03
	Gef.	„ 155, 154	„ 46,45	„ 3,24	„ 9,02.

Schmelzpunkt der Nadeln 284° (k. Th.).

4,546 mg Subst.: 7,755 mg CO_2, 1,38 mg H_2O. — 6,861 mg Subst.: 4,54 ccm n/100-NaOH. — 4,894 mg Subst.: 0,386 ccm N (22°, 748 mm).

Gef. Äquiv.-Gew. 151 C 46,52 H 3,40 N 8,98.

Verseifung der Furan-dicarbon-(2,5)-amidsäure-(2). 540 mg Amidsäure (VII) wurden mit 15 ccm 30%iger alkoholischer Kalilauge versetzt und 60 Minuten am Dampfbad erhitzt; das sich im Laufe der Verseifung ausscheidende Dikaliumsalz wurde in Wasser gelöst, hierauf vom Alkohol abgedampft, der Rückstand mit 2n-Salzsäure angesäuert und die sich ausscheidenden farblosen Krystalle abfiltriert. Die Furan-dicarbon-

[1]) Liebigs Ann. 353, 175 (1907).
[2]) Amer. chem. J. 25, 453 (1901); Chem. Z. 1901, I, 272.

säure-(2,5) wurde scharf getrocknet und in einen Überschuß von Methanol eingetragen, der bei −10° mit trockenem Salzsäuregas gesättigt worden war. Nach 60 Minuten Erhitzen unter Rückfluß wurde am Dampfbad eingeengt, hierauf kalt gestellt, wobei sich ein Krystallbrei abschied, der abfiltriert und aus Wasser umkrystallisiert wurde. Schmelzpunkt des Furandicarbonsäure-(2,5)-dimethylester 109° (k. Th.). Vgl. dazu P. Yoder und B. Tollens[1]), sowie I. Phelps und W. Hale[2]).

15. 20 g β-[Methyl-furyl-(2)]-acrylsäure-amid lieferten 20,1 g (83% d. Th.) Furyl-acrylamid-(2)-carbonsäure-(5) Rohprodukt, bzw. 15,4 g (64% d. Th.) reines Produkt. Die neue Substanz (IX) krystallisiert aus Wasser in farblosen Prismen mit schönen Zwillingsbildungen, oder beim langsamen Ausfallen in langen haarförmigen Nadeln. Schmelzp. 280° (k. Th.) unter Zersetzung. Löslich in viel heißem Wasser, in heißem Methanol, in Alkohol, schwer löslich in Essigester, unlöslich in Äther.

4,208, 4,053 mg Subst.: 8,240, 7,945 mg CO_2, 1,505, 1,445 mg H_2O. — 7,391, 7,297 mg Subst.: 4,14, 4,04 ccm n/100 NaOH. — 4,364, 4,141 mg Subst.: 0,293 (21°, 761 mm), 0,272 (20°, 756 mm) ccm N.

	$C_9H_7O_4N$ (181)			
Ber.	Äquiv.-Gew. 181	C 53,03	H 3,86	N 7,73
Gef.	„ 178, 180	„ 53,40, 53,46	„ 4,00, 3,99	„ 7,83, 7,61.

IV. Synthesen.

1. N-Crotonyl-p-aminophenol. 10,4 g frisch destilliertes Crotonsäurechlorid wurden mit 100 ccm trockenem Äther aufgenommen und bei Zimmertemperatur mit einer ätherischen Lösung von 21,8 g p-Aminophenol unter gutem Schütteln versetzt. Nach wenigen Minuten war kein Säurechlorid mehr zu riechen. Wir ließen das Reaktionsgemisch noch 2 Stunden stehen, filtrierten hierauf den ausgefallenen Niederschlag ab und lösten auf der Nutsche das p-Aminophenolchlorhydrat mit sehr verdünnter Salzsäure heraus. Das Rohprodukt war bereits sehr rein. Ausbeute 17,6 g (99,4% d. Th.). Nach dem Umkrystallisieren aus Wasser schmolz das N-Crotonyl-p-aminophenol bei 189—190° (k. Th.). Löslichkeit und Krystallform vgl. S. 20.

4,033 mg Subst.: 10,01 mg CO_2, 2,20 mg H_2O.

$C_{10}H_{11}O_2N$ (177,1) Ber. C 67,76 H 6,27 Gef. C 67,69 H 6,10.

2. N-Butyryl-p-aminophenol. 17,6 g Buttersäure wurden mit 50 ccm trockenem Benzol und 24 g frisch destilliertem Thionylchlorid versetzt und am Dampfbad unterm Rückflußkühler bis zum Aufhören der Salzsäure-entwicklung erhitzt. Das Reaktionsgemisch wurde hierauf mit einem Fraktionieraufsatz destilliert, wobei wir 5,0 g nicht umgesetzte Buttersäure zurückgewannen. Ausbeute 10,5 g (59,3% d. Th.), Siedep.$_{750}$ 100 bis 101°.

[1]) Ber. chem. Ges. 34, 3453 (1902).
[2]) Amer. chem. J. 25, 452 (1901).

Zu einer ätherischen Lösung von 16,5 g p-Aminophenol fügten wir 9,0 g Buttersäurechlorid hinzu, schüttelten gut durch und filtrierten das Reaktionsprodukt nach 2stündigem Stehen ab. Das Chlorhydrat der Base wurde durch Behandlung des Niederschlages mit Wasser in Lösung gebracht. Ausbeute an N-Crotonyl-p-aminophenol 9,3 g (67% d. Th.). Der Rückstand wurde in Aceton gelöst, mit Tierkohle erhitzt, filtriert, das Lösungsmittel abgedampft und hierauf aus Wasser umkrystallisiert, wobei wir schön gefiederte Nadeln erhielten. Schmelzp. 139—140° (k. Th.).

4,114 mg Subst.: 10,11 mg CO_2, 2,66 mg H_2O.

$C_{10}H_{13}O_2N$ (179,1) Ber. C 67,00 H 7,32 Gef. C 67,02 H 7,23.

Berichtigung: In dieser Z. 242 (1936) ist auf S. 183 und 184 durchweg zu lesen:

nicht Siedep.$_{20}$ sondern Siedep.$_{10}$	nicht Siedep.$_{16}$ sondern Siedep.$_{8}$
„ Siedep.$_{18}$ „ Siedep.$_{9}$	„ Siedep.$_{14}$ „ Siedep.$_{7}$

Auf S. 184, Zeile 4 ist -hexyl- zu streichen.
Auf S. 184, Zeile 9 ist -hexyl- durch -nonyl- zu ersetzen.

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Availability of Energy in Aliphatic Carboxylic Acids by Growing Chicks

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Biological availability of 54 aliphatic acids was compared by mini-test with chicks. Availability of saturated fatty acids with carbon chain between 6 to 12 and of lactic, pyruvic, succinic, L-malic, fumaric and citric acid was satisfactory. Availability of fatty acids with carbon chain shorter than 6 and longer than 12, fatty acids with side-chain, except those of iso-type and unsaturated fatty acids was low. Availability of glycolic, DL-glyceric, DL-malic, and maleic acid, and saturated dicarboxylic acids except succinic acid was also low. Digestibility of saturated fatty acids with carbon chain length longer than 8 decreased inversely with the increase of carbon chain length.

According to the statistics,[1] the demand for feed shown as total digestible nutrients increased linearly at the rate of 0.934 mega-ton per year during the recent 9 years from 9.15 mega-ton in 1959 to 17.06 mega-ton in 1967, while the supply of feed of domestic origin including grass and roughage was increasing steadily but at much slower pace of 0.238 mega-ton per year. The statistics revealed that the trend of rapid increase in demand continued through 1968, and the trend is expected to continue in future.

Because of limited arable area in this country, rapid increase in domestic production of feed can not be expected in near future. Therefore, the large portion of feed supply has to depend upon the import from foreign countries. The statistics also revealed that the amount of feed of foreign origin shown as total digestible nutrients would exceed the domestic production in 1970. In other words, more than half of the supply of feed will come from overseas in 1970 and thereafter. This is really a serious problem for our agriculture as well as for our foreign trade.

With this background on the demand and and supply of feed specific in this country, a 3-year research project was initiated in 1967 to search for new energy sources for domestic animals and poultry under the cooperation of researchers in synthetic chemistry and animal nutrition. Development of the mini-test reported in the previous paper[2] was very encouraging in the planning of the project. The chemicals able to replace the natural energy sources such as cereals may be too expensive at present, but the future progress in research on synthetic process and in mass-production will be effective to make the use

1) "Statistical Year Book on Concentrates," ed. by Livestock Bureau, Ministry of Agriculture and Forestry, Japan; "Pocket Statistics on Agriculture and Fishery," ed. by Agriculture and Forestry Economy Bureau, Ministry of Agriculture and Forestry, Japan.

2) M. Yoshida and H. Morimoto, *Agr. Biol. Chem.*, 34, 684 (1970).

of the chemicals for feed feasible.

Among the 13 synthetic glycols tested in searching for energy sources for poultry feed which are to be supplied without cultivating the land or fishing in the water, 1, 2-propanediol and 1, 3-butanediol were found to be utilzed by growing chicks as dietary energy sources.[3] Rats also were reported to be able to utilize these two glycols.[4,5]

In this paper, the data on the availability of 54 aliphatic acids by growing chicks are presented as the first report of the cooperative 3-year research project, and the possibility of using these chemicals as energy sources in poultry feed is discussed.

EXPERIMENTAL

Test material. Among the materials reported in this paper, 6 dicarboxylic acids, adipic, pimelic, suberic, azelaic, sebasic and undecadionic acid, were synthesized in Oda's Laboratory, Kyoto University. Preparation of mixed acids was a byproduct of industrial production of adipic acid and contained 25 % of succinic, 65 % of glutaric and 10 % of adipic acid. All of the other samples were obtained as reagent, whose grades were shown in Note in Tables I, II and III.

Gross energy of the test materials was determined by an automatic bomb calorimeter, CA-1 manufactured by Shimadzu Seisakusho. Gelatin capsule was used in the determination of volatile materials.

Bioassay. Procedure of the mini-test described in the previous paper[2] was applied to the assays presented in this paper. In all of the assays, a linear dose-response curve was obtained for the growth responses of chicks in 6-day feeding of 3 standard diets of different energy levels, *i. e.* SO-0, SO-4 and SO-8 diet, of which composition was shown in the previous paper.[2] The energy level of the test diet including 5 % of a sample in place of cellulose in the standard SO-0 diet was estimated, based on the growth response of the chicks on the diet, from this dose-response curve. In these dose-response curves x-axis was adjusted as mentioned in the previous paper[2] so that the biologically available energy of the sample could be estimated directly.

Since formic and acetic acid are volatile and are expected to be lost from the diet as in the case of volatile alcohols reported previously,[3] they were infused directly into the crop of chicks as 10% aqueous solution. In this test, the same amount of SO-0 diet was fed to all of the chicks, and the standard dose-response curve was obtained from the growth response of chicks given 0, 0.5 and 1 ml of soybean oil per head daily.[2] Amount of soybean oil corresponding to the growth response of chicks on formic or acetic acid could be calculated from the standard curve, which was easily converted to available energy shown by kcal per g of sample.

Two lots of 2 chicks each or of 4 chicks each were fed either standard or test diets in the standard procedure of mini-test in this Laboratory. However, the number of chicks in each lot was reduced depending on the amount of the sample available. Bioassay was repeated with the same sample if necessary. Total number of the chicks used for each test material was given in Tables I, II and III with experimental data.

In most of the tests, growth rate, feed intake and mortality of the chicks were recorded and, if necessary, a sample of excreta was collected to determine the digestibility of the sample as mentioned later. To compare the difference in palatability of the samples tested at different times, average feed intake of the test diet was shown by a ratio of the intake to that of the standard SO-4 diet as palatability index, in which the intake of SO-4 diet was taken as 100.

Appropriateness of the test material to be used as poultry feed was judged mainly on the availability of energy, *i. e.* ratio of available energy to gross energy; mortality; palatability; and digestibility.

Determination of digestibility. When the determination of digestibility was necessary, chromic oxide at 0.05 % level was mixed in all of the standard and test diets before feeding to the chicks and the excreta of the chicks were collected for the last 2 days of mini-test. Contents of crude fat and chromic oxide in the diets and excreta were determined by ether extraction with Soxhlet apparatus and with phosphate

3) M. Yoshida, H. Hoshii and H. Morimoto, *Japan Poultry Sci.*, 6, 73 (1969).
4) K. Ashida, private communication, (1968).
5) S. A. Miller and H. A. Dymsza, *J. Nutr.*, 91, 79 (1967).

reagent,[6] respectively. True digestibility of the test material was calculated as follows:

$$DC = 100 - (FF_T \times DC_T / FC_T - FF_S \times DC_S / FC_S) / (DF_T - DF_S) \times 100$$

where, DC: digestibility coefficient (%)
FF_T: fecal crude fat on test diet (%)
FC_T: fecal chromic oxide on test diet (%)
DF_T: dietary crude fat of test diet (%)
DC_T: dietary chromic oxide of test diet (%)
FF_S: fecal crude fat on SO-0 diet (%)
FC_S: fecal chromic oxide on SO-0 diet (%)
DF_S: dietary crude fat of SO-0 diet (%)
DC_S: dietary chromic oxide of SO-0 diet (%)

This procedure was only applicable to the test materials soluble in ethyl ether.

RESULTS AND DISCUSSION

It should be pointed out before dicussing the data obtained in these mini-tests, that the procedure was devised to be suitable to estimate biological availability of small amounts of samples, *i.e.* 5 to 50 g, sacrificing the length of feeding period and the number of chicks,

TABLE I. GROSS ENERGY, AVAILABILITY, PALATABILITY AND DIGESTIBILITY OF SATURATED FATTY ACIDS

Sample		No. of chicks	Gross energy kcal/g	Available energy kcal/g	Availability AE/GE %	Palatability index[c]	Digestibility coeff. %	Note
Formic	C_1	4/4[b]	—	x[c]	—	30		Special[d]
Acetic	C_2	1/4	3.15	x	—	35		〃
Propionic	C_3	4	4.79	−6.4	—	57		〃
n-Butyric	C_4	4	5.80	−19.4	—	44		First[d]
i-Butyric	C_4	4	5.78	3.5	61	80		Special
n-Valeric	C_5	12	6.53	0.8	13	68		First
i-Valeric	C_5	12	6.45	−6.3	—	71		Special
Pivalic	C_5	2	6.61	−22.6	—	57		BDH[d]
n-Caproic	C_6	12	7.36	5.0	68	79		First
i-Caproic	C_6	12	7.15	5.0	70	81		Reagent[d]
n-Heptoic	C_7	12	7.59	6.9	90	73		BDH
n-Caprylic	C_8	20	7.99	3.1	39	69	100	Special
2-Et-Hexanoic	C_8	1/4	7.97	x	—	13		First
n-Nonanoic	C_9	9	8.16	6.8	84	65	97	BDH
n-Capric	C_{10}	4	8.48	9.1	107	52	94	First
n-Undecanoic	C_{11}	2	8.55	8.0	94	72		Special
Lauric	C_{12}	4	8.75	9.2	105	81	83	Reagent
Myristic	C_{14}	4	9.13	3.0	33	104	37	Special
Palmitic	C_{16}	4	9.30	1.4	15	105	34	〃
Stearic	C_{18}	4	9.45	0	0	106	38	〃
Behenic	C_{22}	4	9.73	−5.1	—	100		Reagent

a) See text.
b) Dead/total
c) Can not be estimated because chicks died.
d) Special: special grade reagent; First: first grade reagent; Reagent: reagent; BDH: obtained from the British Drug Houses Ltd.

6) M. Yoshida, K. Kosaka, S. Horii and K. Kameoka, *Japan. Poultry Sci.*, 4, 24 (1967).

therefore, the accuracy of the conclusion.[2] The mini-test is certainly a useful tool for evaluating a sample for screening purpose, in other words, for judging whether or not the sample is hopeful as dietary energy source for poultry. Therefore, it is most necessary to confirm the conclusion of the mini-test by long term feeding experiments with a large number of chicks.

The data of the mini-tests on saturated fatty acids were summarized in Table I.

It was unexpected that chicks could not utilize energy in most of the volatile fatty acids with carbon chain shorter than 5, which are known to be produced in rumen and readily utilized as energy source by ruminants. Since acetic acid, as well as formic acid, was infused in crop directly as 10% aqueous solution,[2] physical and chemical properties, such as pH, may have physiologically detrimental influence and may lower the growth rate as well as palatability of the diet. On the other hand, propionic and butyric acid were mixed into feed and fed to chicks. Low palatability of these acids may suggest that physical and chemical natures, such as strong offensive smell or acidity of the samples make the chicks lose appetite, resulting in slow growth rate. However, growth retardation of the chicks shown by negative value of available energy seemed to be too severe to be explained by small appetite, since the effect of difference in feed intake on the available energy was mostly cancelled out by taking body weight gain per 100 g feed instead of body weight gain itself in these mini-tests. Further studies are certainly necessary to confirm whether chicks can actually utilize volatile fatty acids or not, and to show the reason why they do not if it is confirmed that chicks have no ability to utilize volatile fatty acids. Availability of iso-butyric acid by chicks seemed to be higher than that of the other volatile fatty acids. The observation should be confirmed in further studies.

Ozaki's old work[7] revealed that rats on the diets containing 10% of acetic, butyric, caprylic and capric acid died within 40 days of experimental period, although his experimental diet may have been too simple from the present knowledge of rat nutrition. Ashida[8] found that growth rate of rat on 5% acetic, propionic and valeric acid was satisfactory, although none of them exceeded the growth rate of the control rats. Those findings on both rats and chicks suggest that some problems have been left over in the attempt to utilize volatile fatty acids as feedstuff for energy source.

Fatty acids of odd numbers of carbon chain: heptoic, nonanoic, and undecanoic acids, all of which are not natural, were available by the chicks as efficiently as the natural even number fatty acids. The data may encourage us to study further the possibility of using synthetic fatty acids for feed, which may be a mixture of fatty acids of odd and even numbers of carbon.

The data on pimelic and 2-ethyl-hexanoic acid suggested that chicks could not utilize fatty acids with side chain, except that of iso-type, *i.e.* single methyl branch at the opposite end of carbon chain to the carboxyl group. The findings may give severe restriction to the expectation in using synthetic fatty acids as feed, since such acids should be composed of only normal and iso-type acids.

Chicks could utilize free fatty acids with carbon chain length between 6 to 12, but it should be pointed out that the palatability of these acids was as low as less than 81, and at the same time specific offensive smell of these acids should be kept in mind since it is certainly unpleasant to handle the acids as feedstuff.

Availability of energy in fatty acids of carbon chain length longer than 14 is lowered with the increase in chain length as shown in

7) J. Ozaki, *Nippon Nōgeikagaku Kaishi*, 8, 1286 (1932).
8) K. Ashida, private communication (1969).

Table I. Low availability of these fatty acids is mainly due to low digestibility as shown in the column of digestibility coefficient in Table I. Palatability index higher than 100 by 4 to 6 on these fatty acids indicated that chicks took 4 to 6% more feed which contained 5% of an undigestible, consequently inert fatty acid than the control SO-4 diet. The findings agree with those reported by Renner and Hill,[9] who studied the utilization of free fatty acids of carbon chain length between 12 to 18. They observed that absorbability of saturated fatty acids by both growing chicks and laying hen decreased with the increase in carbon chain length, while the absorbability of oleic acid was satisfactorily high. They also reported low palatability of lauric acid and suggested that lauric acid might have some irritating effect on the gastro-intestinal tract of chicks. This irritating effect may not be specific to lauric acid, but the effect seems to be rather common to the free saturated fatty acids of carbon chain length from 3 to 12 as shown by the palatability index in Table I.

The data on unsaturated, hydroxy and keto acids were summarized in Table II.

Though no chick died on glycolic acid as shown in Table II the chicks took feed at the level of only 32% of the amount taken by chicks on the standard SO-4 diet, and they lost body weight. Availability and palatability of lactic acid were satisfactorily close to those expected from the biochemical glycogenic function. Availability of energy in pyruvic acid was also satisfactory, although paratability was somewhat low and the acid gave offensive smell to the diet. On the other hand, availability of DL-glyceric acid, D-form of which is a constituent of anaerobic muscle glycolysis, was unexpectedly low. Antagonistic effect of L-form to the utilization of D-form may be one of the possible explanation of low availability.

Table II. Gross Energy, Availability and Palatability of Unsaturated, Hydroxy and Keto Acids

Sample		No. of chicks	Gross energy kcal/g	Available energy kcal/g	Availability AE/GE %	Palatability index[a]	Note[a]
Glycolic	C_2	4	2.18	—[b]	—	32	Reagent
Lactic	C_3	8	3.23	2.4	76	97	Special
DL-Glyceric	C_3	2	2.87	0.1	3	86	Reagent
Pyruvic	C_3	8	3.06	3.8	123	88	Special
Acrylic	C_3	2/4[a]	3.68	x[a]	—	22	Special
Methacrylic	C_4	4	5.39	−37.6	—	46	Reagent
Crotonic	C_4	4		−33.4	—	41	Reagent
Sorbic	C_6	4	6.58	3.2	50	66	Special
Gluconic	C_6		1.69	−2.3	—	98	Reagent 50% solution
Undecylenic	C_{11}	2	8.31	−5.4	—	52	Reagent
Elaidic	C_{18}	4	9.27	−5.7	—	78	BDH
Linoleic	C_{18}	4	9.32	−19.8	—	54	Reagent
Linolenic	C_{18}	1	9.12	−37.0	—	20	Special.

[a] See footnotes of Table I.
[b] Chicks lose body weight.

9) R. Renner and F. W. Hill, *J. Nutr.*, 74, 259 (1961).

TABLE III. GROSS ENERGY, AVAILABILITY AND PALATABILITY OF DI-, TRI-CARBOXYLIC ACIDS

Sample		No. of chicks	Gross energy kcal/g	Available energy kcal/g	Availability AE/GE %	Palatability index[a]	Note[a]
Malonic	C_3	4	1.94	−12.5	—	66	Special
Succinic	C_4	12	2.99	5.1	173	90	〃
DL-Malic	C_4	4	2.37	−8.9	—	61	〃
L-Malic	C_4	4	2.44	6.4	262	47	Reagent
Tartaric	C_4	4	1.85	0.9	49	77	Special
Maleic	C_4	4/4[c]	2.77	x[c]	—	7	〃
Fumaric	C_4	4	2.78	5.1	183	82	〃
Oxalacetic	C_4	4	2.08	−9.4	—	41	BDH
Glutaric	C_5	2/4	3.90	x	—	11	Special
α-Ketoglutaric	C_5	4	2.90	−4.8	—	37	〃
Itaconic	C_5	1/4	3.58	x	—	20	Reagent
Adipic	C_6	4	4.50	−35.3	—	52	[b]
Citric	C_6	4	2.21	2.1	95	86	Special
cis-Aconitic	C_6	4	2.89	−15.1	—	27	Reagent
Pimelic	C_7	1	4.81	−16.2	—	87	[b]
Suberic	C_8	4	5.59	−16.0	—	62	[b]
Azelaic	C_9	4	5.93	—[c]	—	34	[b]
Sebacic	C_{10}	1		−32.6	—	73	[b]
Undecadioic	C_{11}	2	6.66	−14.7	—	49	[b]
Mixed acids[d]		8	3.89	0.7	18	60	

[a] See footnotes of Table I.
[b] See text.
[c] See footnote 2 of Table II.
[d] Contained 25% of succinic acid, 65% of glutaric acid and 10% of adipic acid.

All of the unsaturated acid, except sorbic acid, depressed growth of chicks as shown by negative available energy in Table II. Rancidity of these acids may be one of the reasons of ill effect. Acrylic acid was fatal to the chicks.

The data on di- and tri-carboxylic acids were summarized in Table III. Among the di- and tri-carboxylic acids tested, succinic acid, L-malic acid, fumaric acid and citric acid were readily available by the chicks, although their palatability was not quite satisfactory, being less than 90% of feed taken by chicks on the standard SO-4 diet. It should be pointed out that all of these acids are the constituent elements of the tri-carboxylic acid cycle in aerobic metabolism of carbohydrate. On maleic acid, the isomer of fumaric acid, chicks took only 6 g of diet at the beginning of the experiment, when they were starved, and all of them died within two days. Chicks can be alive as long as 6 days even under the conditions of losing body weight, as in the case of feeding glycolic acid shown in Table II and azelaic acid shown in Table III. No group of chicks except those fed maleic acid died within 2 days on various poisonous materials shown in Tables I, II and III. These observations indicated that maleic acid worked out as a strong poison. It is expected that maleic acid, as an antagonist of fumaric acid, breaks down the tri-carboxylic acid cycle and cuts off the supply of biologically available energy from

TABILITY

...bility ...GE	Palatability index[a]	Note[a]
–	66	Special
3	90	"
–	61	"
2	47	Reagent
9	77	Special
–	7	"
3	82	"
–	41	BDH
–	11	Special
–	37	"
–	20	Reagent
–	52	b)
5	86	Special
–	27	Reagent
–	87	b)
–	62	b)
–	34	b)
–	73	b)
–	49	b)
3	60	

...dipic acid.

On maleic acid, the isomer ...hicks took only 6 g of diet ... of the experiment, when ...d, and all of them died ... Chicks can be alive as long ...der the conditions of losing ...n the case of feeding glycolic ...ble II and azelaic acid shown ...No group of chicks except ...acid died within 2 days on ...s materials shown in Tables ...hese observations indicated ... worked out as a strong ...ected that maleic acid, as ... fumaric acid, breaks down ... acid cycle and cuts off the ...ically available energy from

carbohydrate. Unexpectedly strong detrimental effect of malonic acid, and especially of glutaric acid might be explained by the antagonistic effect of these acids to those related to the tricarboxylic acid cycle of aerobic metabolism of carbohydrate. Detrimental effect of DL-malic acid was also suspected to be due to the antagonistic effect of D-isomer, as in the case of DL-glyceric acid.

Rats were also found to utilize fumaric acid efficiently, but they lost body weight severely on maleic acid.[8] Availability of the saturated dicarboxylic acids of carbon number from 3 to 11, except 4, *i.e.* succinic acid, was also very low according to Ashida's data[8] on rats. Ozaki[10] also found that rats on azelaic acid grew slowly and their body weight was decreased in the latter half of feeding period of about 30 days.

Negative availability of oxalacetic acid, α-ketoglutaric acid and *cis*-aconitic acid, all of which are the constituents of tricarboxylic acid cycle, were unexpected. However, it should be kept in mind that these acids were given to chicks orally as a mixture with other dietary ingredients, including protein and minerals. Therefore, some chemical change may have possibly taken place in the diet which decomposed the acids themselves or other dietary nutrients possibly producing detrimental substances in the diet. Different results will be obtained if these acids be given with some protecting means, or given separately from the other dietary ingredients.

Growth depression and death of chicks on itaconic acid, *i.e.* ethylenesuccinic acid, may suggest either that the acid works as an antagonist of succinic acid or the related compounds disturbing the tricarboxylic acid cycle as in the case of maleic acid and glutaric acid, or that the acid becomes rancid in the diet as in the case of unsaturated fatty acid shown in Table II. It should be pointed out that the chemical structure of itaconic acid, *i.e.* ethylenesuccinic acid, is related to that of acrylic acid, which is ethyleneacetic acid, and of metacrylic acid, which is ethylenepropionic acid. Acrylic acid was toxic and metacrylic acid depressed growth rate as shown in Table II.

Dibasic acids of carbon chain longer than 6 were all unavailable for chicks. Enzymic system to utilize energy of these dibasic acids seemed to be lacking in chicks' body. Rats also were unable to utilize these acids. Since these dibasic acids are crystal and, therefore, readily mixed in the poultry feed which is served as mash, great expectation was given to these acids. However, chicks dislike these dibasic acids as shown by low palatability index and can not utilize energy of these acids efficiently. As for succinic acid, only one hopeful energy source among dibasic acids tested, its relatively low energy level, 2.99 kcal/g, may be a weak point for future use of this acid as dietary energy source economically in large scale.

Mixed acids shown in Table III were a by-product of industrial production of adipic acid, of which low availability certainly corresponded to the low availability of glutaric acid, the major constituent of this mixed acids.

10) J. Ozaki, *Nippon Nogeikagaku Kaishi*, 3, 977 (1927).

A germination test for the non-specific detection of preservatives.

By F. Baum and Chr. Wardsack.

1. Introduction.

The use of preservatives in foods is regulated by law in the individual countries. This regulation relates to the type of preservative, the admissible maximum addition, and the food released for preservation. The object of the addition of chemical preservatives is to make the food stable by this addition, that is, to prevent the micro-organisms contained therein from multiplying, at least for a certain time. From investigations by v. Schelhorn (1) of the effect of salicylic acid, benzoic acid, formic acid and sulfurous acid on willia anomala it is known that a permanent prevention of multiplication is not possible but that the micro-organisms contained therein are either destroyed or increased. It can happen with low concentrations of the added preservative that multiplication only sets in after weeks. The causes are assumed to lie in resistance mutations or in the decomposition of the preservative. These results found by v. Schelhorn (1) correspond to the experiences which are obtained again and again in the storage of preserved foods. In most cases spoilage of the foods starts after weeks or months.

This finding about the effect of the preservatives also includes the possibility of abandoning the legal basis in the preservation of foods in order to either exceed the permissible maximum dose or to use prohibited agents for the preservation. These "illegal" preservatives are more or less toxic, and among them are even highly toxic compounds, like the derivatives of bromoacetic acid, complex mercury compounds and others, as reported by Moseel(2). Besides, it is possible that higher doses of the preservatives get into the food by carelessness. Finally the possibility of an illegal preservation may result from interstate commerce, since the legislation on preservatives varies in the different countries.

For these reasons it is necessary to subject the foods to controls in order to protect the consumer from health injuries. In view of the great number of compounds that can have a preservative effect, it is difficult to find an un-

known existing agent with chemical methods. This task would be extremely difficult and cumbersome and could not be solved even by using the methods of infrared spectrophotometry and chromatography. The only thing left is the microbial test. The latter is based on the fact that certain micro-organisms, which are combined with a food, for example, with an extract prepared therefrom, show normal growth or typical physiological reactions, while an inhibition sets in when the food contains effective amounts of preservatives. The microbial test is a non-specific method which only permits to determine whether an active component is present or not. A great number of articles have appeared in the literature about the non-specific detection of preservatives in foods. It is not our object here to discuss all these methods and to weigh their advantages and disadvantages. Those interested will find a comprehensive survey of the various possible applications of microbial methods in the article by Mossel (2). There an extensive bibliography wil also be found.

The fermentation test according to Kluyver seems to be the most common detection method. Various modifications of the test were indicated by Mossel (2), Coretti (3), Mossel and De Bruin (4) and Mossel, Mandersloot and Gentis (5), which relate partly to the extraction of the active substances, if the food itself can not be used as a medium, for example, meat. The Kluyver test, which uses a gasometric technique, is based on the principle that the food to be tested is fermented in the simplest case directly after the addition of certain amounts of nutrients and yeast cells. The reaction is read after 24 hours in the intervals of 3 hours by measuring the amount of CO_2 formed from dextrose by the fermentation of the yeast. The reaction is considered completed when the maximum amount of gas has formed in a preservative-free extract. If no fermentation has taken place after 48 hours, it is concluded that preservatives are present. There is a suspicion of the presence of preservatives if no or a delayed gas formation has taken place after 33 hours, but maximum fermentation appears after 48 hours.

A similar detection method based on the fermentation test is described by Koch and Schaffner (6) for the non-specific detection of chemical preservatives

in fruit juices (sweet cider), non-alcoholic beverages, and fruit concentrates. But here there is a certain methodological difficulty. 100 mg% p-oxybenzoic acid manifest themselves after 15 hours of incubation as delayed fermentation. After 24 hours the fermentation should be normal again. Rather strange seems the likewise mentioned "mold fungus test." Here 25 ml cider are poured into sterilized Petri dishes and the dishes are exposed without cover for 60 minutes to "accidental infection" by room air. After incubation at 25 deg.C for three days it is assumed in the evaluation of grown mold fungus cultures that the cider is preservative-free. But with this method it is not even possible to detect a positive infection, apart from the species of the mold fungi. Another test is based on the growth of candida brumptii and is described by Mossel, Zwart and De Bruin (7). From the type of growth of the yeast cells on agar plates which contain the food to be preserved in homogenized form, the presence of absence of preservatives is assumed.

Bernarts (8) uses the osmophilic yeast saccharomyces rouxii for the non-specific detection of preservatives in jam. Sandi and Szantha (9) work according to the Kluyver-Mossel method with saccharomyces cerevisiae but with a different fermentation tube, which is said to be suitable for systematic series-investigations. Del Campo (10) weighs the amount of CO_2 formed in the fermentation test with saccharomyces cerevisiae.

After this brief enumeration of only a few methods, we will also mention the suitability in the routine method. In our opinion a test method which is suitable for series investigations should meet the following requirements:

1. The test must be simple to realize, that is, without complicated technical and optical apparatus.
2. The test result must be available not later than on the following day, that is, after 24 hours.
3. Handling of the samples must not require much time.
4. Even small amounts of preservatives must be detectable.
5. It should be possible to identify preservatives contained even in food residues, for example 1-2 ml of a liquid food.

6. The glass vessels used must be easy to clean.

7. The necessary microbiological operations can be carried out even by workers who are not trained in this field.

If we apply these viewpoints to the above mentioned methods, we can say that the Kluyver test, variously modified, lasts too long with a test period of 48 hours. Besides, the fermentation saccharometers used according to Einhorn are very difficult to clean (it is possible that the fermentation tubes described by Coretti (3), which were not used by us, were easier to clean. (We already reported briefly about our experiences regarding the accuracy of this test (Baum and Lamm (11)). It is not easy to separate clearly positive and negative results, since great differences appear partly in parallel batches. The method described by Koch and Schiffner (6) has apart from the above mentioned disadvantages, the difficulty that the results are read after 15 hours. This does not go well with a working day of 7½ or 8 hours. The "mold fungus test" yields more or less accidental results and must therefore be eliminated. Our requirement of the detectability of preservatives even in minute food residues is not met by any of the above mentioned methods. The agar diffusion test, which was to be used by us (Baum and Lamm (11) for the detection of preservatives, is unsuitable for routine work and is practically only of theoretical interest.

In working out a method which must withstand the stress of series-investigations, we started fromthe consideration that this test should be carried out with a minimum of energy. Profitability also plays a certain part. But the 7 conditions listed above are considered by us the main points.

2. Execution of the germination test.

The impetus for working out a germination test came, apart from our own considerations, from studies by Raible (12) and Rehm (13). Raible writes about "a simple method for testing the effectiveness of chemical preservatives against mold fungi", and Rehm reports his experiences in the application of this method. The test is carried out as follows:

Filter paper disks are saturated with the various dilution-stages of the inhibitor in the nutrient medium and inoculated with spores and hyphae-materials laid out in a Petri dish (moist chamber). The organisms used were aspergillus niger and fusarium nivale. The development time of the fungi during the incubation was 3-4 days. After this time the samples were evaluated by reading the development of the mycelia or by the iodine test. As advantages of the method are mentioned savings in space and materials and greater precision. If we want to use this method for detecting preservatives in foods, the long, incubation period must be considered a disadvantage.

We started from the consideration that the criterion is not the frowth of the mycelium, but the germination of the fungus spores. Since the germination takes place under certain conditions within 24 hours, the test period up to the evaluation can be considerably reduced.

For the observation of the germination of fungus spores it is customary to effect the culture in so-called "hanging drops". This method is naturally not suitable for a routine investigation. We worked therefore without cover glass and pipetted 0.05 ml of the test solutions into the hollow ground section of the object slides and incoulated with some spore material of the mold fungus aspergillus orycae. These prepared slides were placed in large Petri dishes in which round filters were arranged which were saturated with 6% NaCl solution. Since the test solutions were standardized to a sugar content of about 10%, we obtained a moist chamber after covering the dishes. This way the drops do not dry out nor is water attracted, so that the preservative concentration remains understandably constant. The tests were incubated for 24 hours at +30 deg.C and evaluated after this time by subjecting the drops contained in the hollow ground section of the slides to a microscopic examination under low magnification. The germination can be recognized by the formation of germ tubes. In most cases a microscopic examination is not necessary, because a grown dense mycelium can already be recognized with the naked eye if the test is negative. Sommer and Halbsguth (14) interpret the formation of vacuoles in the interior of phycomyces sporangiospores as germination. But this criterion can not be

used in the high-speed routine operation, so that we consider here the formation of germ tubes as the criterion. These germ tubes have frequently a different appearance, depending on the preservative used (see fig.1-8).

It can happen that not all spores, or only a few conidia spores have germinated. We considered a percentage of over 50% germinated spores as positive. If only a few spores have germinated, this is considered "negative" or "not germinated". The check of the test batches is extremely short, since its brief survey suffices to determine a positive germination. It is not necessary to count or measure. A germination which is visible after an incubation period of 24 hours is interpreted as "no preservative in the solution" or "no effective amount of preservative in the solution". If the germination of the spores has not started within 24 hours, this is evaluated as an "effective amount of preservative in the solution". The nutrient solution used in the test, modified according to Haehn (15), has the following composition:

2.5 g NH_4NO_3
1.0 g KH_2PO_4
0.25 g $MgSO_4$ pH = 2.5
100.g saccharose

Fig. 3. Germinated conidia spores of aspergillus orycae. Preservative: sorbic acid 5 mg%. Magn. ab. 300x.

Fig. 4. Germinated conidia spores of A. orycae. Preservative nipagin A 10 mg%. Magn. ab. 300x.

Fig. 5. Germinated conidia spores of A. orycase. Preservative: Na-benzoate 2.5 mg%.

Fig. 6. Germinated conidia spores of A. orycae. Preservative: Nipasol 5 mg%.

Fig. 7. Start of germination of conidia spores of A: orycae. Preservative: benzoic acid 5 mg%.

Fig. 8. Germinated conidia spores of A. orycase. Control without preservative.

Fig. 1. Ungerminated conidia spores of A. orycae. Magnification ab.300x.

Fig. 2. Germinated conidia spores of A. orycae. Preservative: Na-sorbinate 25 mg%.

The solution is sterilized for 30 minutes in Koch's digester. In this nutrient-solution the various preservatives are dissolved and corresponding dilution series are prepared. Of particular importance is that the pH-value is standardized to 2.5 ± 0.1. This low pH-value was deliberately selected since the active component in the preservative is the undissociated molecule according to v.Schelhorn (16) and the undissociated portion of most preservatives increases with dropping pH-value. 0.05 ml of the nutrient solution with or without preservative are placed on the hollow ground slides, as mentioned above. 3 parallel samples are prepared and inoculated with a few spores of A. orycae. These spores are picked up with a stab needle from the culture plate of the mold fungus and placed in the drops of the test solution contained in the hollow ground sectinn. The mold fungus A.orycae is grown on grits agar of the following composition:

biomalt	60 g
peptone	5 g
NACl	5 g
glycerin	5ml
agar	30 g
nutrient bouillon	1 l

The nutrient soil with a pH of 7.2 is boiled briefly in the digester and filtered subsequently. The sterilization time is 30 minutes at 121 deg.C in the autoclave.

The plates incoulated with A. orycae were incubated at 30 deg.C. The inoculated test batches were put into Petri dishes, as described above, which contained a filter paper saturated with a 6% NaCl solution, and incoulated at 30 deg.C for 24 hours. Subsequently the slides were checked. It must be pointed out that Petri dishes, filter paper, as well as the hollow ground slides neet not be sterile, so that this operation can be eliminated in the germination test; the nutrient solution need not be sterilized either. We sterilized the nutrient solution only in order to make it more stable over a

At this point it should be mentioned that the growing of the mold fungus is very simple even for an unskilled worker. In order to simplify the test further we preserved spores so that the growing of the test organism can be completely eliminated. Corresponding tests are almost completed and will be reported later.

3. On the use of A. orycae as a test organism.

First tests with A. niger conidia spores showed no satisfactory results. The reasons is probably that A. niger, at least the strains used by us, germinate even in the presence of high doses of preservatives, which means they are relatively resistant and capable of decomposing preservatives.A. orycae as a test organism, on the other hand already reacts to small amounts of preservative and has proved highly satisfactory in the determination in almost three years of use.

4. Results of the method.

Various chemical preservatives have been tested so far with the germination test. In order to determine the limiting inhibition concentration, the preservatives were tested in different concentrations. The substances used were both legal and illegal preservatives. The following substances have been tested so far:

sorbic acid	dehydracetic acid
sodium sorbinate	hexamethylene tetramine
benzoic acid	formic acid
sodium benzoate	nipasol
salicylic acid	nipagin A
sodium salicylate	sodium nitrite
boric acid	table salt

The difficultly soluble acids and esters were treated in the waterbath at +50 deg. to enhance the dissolution.

Tests with nutrient solution without preservative showed that the germination of the spores took place in this medium after 10 hours incubation at 30 deg.C. The setting of the incubation period at 24 hours was arbitrary.

This time seemed to us optimal in a routine method, since the evaluation was then effected at the same time of day, so that a mixup of the samples was almost impossible.

The following limiting inhibition concentrations were obtained in the germination test

Detectable amounts of preservatives (delay of germination)

sorbic acid	10 mg%
Na-sorbinate	30 - 50 mg%
benzoic acdi	10 mg%
Na-benzoate	10 mg%
salicylic acid	1.0 mg%
Na-salicylate	2.5 mg%
boric acid	750 - 1000 mg%
dehydracetic acid	10 - 25 mg%
hexamethylene tetramine	200 - 250 mg%
formic acid	200 - 250 mg%
nipasol	10 mg%
nipagin A	15 - 20 mg%

If we evaluate the negative result of the individual samples of parallel batches (there is a certain transition range from "germinated" to "not germinated" which manifests itself by the germination of about 50% of the sowed-in spores of "+" and "-" of the individual samples) we obtain the following results:

Detectable amounts of preservatives:

benzoic acid	2.5 - 5.0 mg%	dehydracetic acid	5.0 - 10.0 mg%
Na-benzoate	5.0 - 10.0 mg%	nipasol	2.5 - 5.0 mg%
salicylic acid	0.25 - 0.5 mg%	nipagin A	5.0 - 10.0 mg%

The presence of table salt up to 5 g% as well as of 1 g % sodium nitrite does not interfere with the test.

If we compare the values with the results by Koch and Schiffner (6) and Coretti (3), who all use the fermintation test, we find the following:

Detachable amounts of preservatives in mg%.

Preservative	Germination Test	Accord. to Coretti	Accord. to Koch and Schiffner
benzoic acid	10	25-50	10-30
sodium benzoate	10	25-50	15-20
Nipasol	10	x	25-75
Nipagin A	15-20	100-500	40-120
Formic acid	200-250	50-100	150-200
Dehydracetic acid	10-25	x	25-50
Sorbic acid	10	x	25-50
Na-salicylate	2.5	25	x
Boric acid	750-1000	100-200	x

x - no data

The indicated values show that, with the exception of formic acid and boric acid, the other preservatives can be detected in the germination test in lower concentrations than in the fermentation test. This is not changed by the fact that Coretti dissolved the preservatives in ham extract, while Koch and Shiffner dissolved them in apple juice, because we found our indicated limiting inhibition concentrations in apple juice, for example, as it will be shown in a later study.

5. Dependence of the testing method on the pH-value, time and age of spores.

As mentioned above, it is important to work at a possibly low pH-value in order to fully utilize the effectiveness of the preservative. According to v. Schelhorn (16) the effectiveness of the preservatives depends to a great extent on the raction of the system substrate + preservative. All these compounds are to a great extent ineffective in a weakly acid to neutral reaction, or they are only effective in large amounts. It can therefore be concluded that only the undissociated acid molecules are responsible for the preservative action.

The following table (table 1) (from v.Schelhorn (16)) is reproduced here to show how the undissociated portion and thus the effectiveness increases

with decreasing pH-value in some acids.

Table 1. Dissociation of various preservatives (accord. to v. Schelhorn (16).

Preservative	Dissociation	Undissociated portion in % at pH 2	pH3	pH4	pH5	pH 6	pH7
Salicylic acid	$1.06 \cdot 10^{-3}$	90	49	8.6	0.94	0.094	0.0094
Formic acid	$2.1 \cdot 10^{-4}$	98	83	32	4.5	0.47	0.048
p-chlorobenzoic acid	$9.3 \cdot 10^{-5}$	99	94	52	9.7	1.06	0.107
Benzoic acid	$6.6 \cdot 10^{-5}$	99	94	60	13	1.5	0.15
Propionic acid	$1.4 \cdot 10^{-5}$	99.9	99	88	42	6.7	0.71

If we consider the indicated value, we see that the pH-value of 2.5 of our nutrient solution is extremely valuable. The use of A. orycae as an acidophilic micro-organism must also be considered an advantage in this connection.

For adjusting the pH-value, we used hydrochloric acid and pH paper Stuphan with a test range of pH 1.2 - 217 in graduations of 0.3 units. It is important that the pH-value is exactly standardized to 2.5 + 0.1, otherwise no comparable results are obtained, as shown in table 2.

Table 2. Detectable amounts of preservatives in dependence on the pH-value (test period 2½ hours, incubation temperature: 30 deg.C) in mg%.

Preservative	pH 2.5	pH 3.0	pH 4.0
Sorbic acid	10	10	25
Na-sorbinate	30 - 50	50	>200
Benzoic acid	10	10	25
Na-benzoate	10	10	100
Salicylic acid	1.0	2.5-5.0	25
Na-salicylate	2.5	5.0-10.0	>100

It can be seen clearly from this table that the inhibiting effect on A. orycae diminishes with increasing pH-value. The difference between pH 3.0 and pH 4.0 is particularly noteworthy.

The test period was set by us at 24 hours, as mentioned above. At this time the spores of the control (without preservative) have already germinated

and a visible dense white mycelium has formed which is visible even without the microscope. In order to clarify the question whether ungerminated spores have lost their capacity to germinate after 24 hours of incubation under the action of the preservative dose or whether the germination has been delayed, we evaluated tests, using different preservatives, after an incubation period of 24 and 48 hours. The results are compiled in table 3.

Table 3. Germination of the conidia spores of A. orycae after 24 and 48 hours of incubation at 30 deg.C.

Preservative		24 hours	48 hours
Na-sorbinate	25 mg%	+ + +	
Na-sorbinate	50 mg%	- - - -	+ + $\bar{+}$
Benzoic acid	5 mg%	+ + +	
" "	10 mg%	- - -	+ + +
" "	25 mg%	- - -	+ + +
Na-benzoate	10 mg%	- - -	+ + +
Na-salicylate	2.5 mg%	- - -	- - -
Salicylic acid	0.5 mg%	+ + -	+
" "	1.0 mg%	- - -	- - -

Explanation: 3 parallel samples in each test.

+ = germinated

- = not germinated

$\bar{+}$ = about 50% of the spores germinated

It was found that the limiting inhibition concentrations indicated by us apply only to an incubation period of 24 hours. But this time must be absolutely maintained in the test. After 48 hours spores have partly germinated which are exposed to a higher preservative dose. This is particularly conspicuous in benzoic acid. But the addition of 2.5 mg% Na-silicylate or 1.0 mg% salicylic acid still prevents germination even after an incubation period of 48 hours. But this does not mean that the spores have lost their germinating capacity, since the test was stopped after this time. A further delay of the germination is thus possible. The conidia spores used for the test

were taken from 3-6 days old cultures of A. orycae. No difference was found in the germinating capacity. Tests with older spores are still being carried out to check the possibility of using preserved spores, as mentioned above.

6. Discussion

The germination test described here, using A. orycae as a test organism, is in our opinion suitable for the series investigations for the non-specific detection of preservatives in foods. The germination test has several advantages over other methods. Thus, small amounts of preservatives can already be detected in most cases. This is probably due to the low pH of 2.5, which is particularly favorable for the investigation since most preservatives are more effective as this pH-value than at pH 4.0, which is used in the fermentation test. Another advantage is that only small amounts of foods are required for the test (1 ml of a liquid food is quite sufficient) and the result of the test is already available after 24 hours. The setting of the incubation period at 24 hours excludes to a great extent wrong evaluation periods, since the evaluation is effected at the same time of the day as the preparation of the samples, only one day later. The reading of the results is only made once, and need not be repeated as in the fermentation test. The evaluation is effected under low magnification under the microscope. If no effective amount is contained in the preservative, a microscopic examination is not necessary, since the grown mycelium is then visible without the microscope. An advantage is also that the glass utensils required (Petri dishes and hollow ground object slides) are inexpensive and economical in use and can be easily cleaned. Sterilization of the glass utensils and of the test solutions is not necessary.

A disadvantage of the germination test is still that microbiological growing is necessary. Growing A. orycase is easy and does not present any difficulties even for microbiologically unskilled workers, but in the chemical laboratory it frequently interferes with current operations since it is necessary to work here under sterile conditions. If our investigations of the use of preserved spores should be successful, the germination test will be considerably simplified. The adjustment of the test substrate to a sugar con-

tnet of ab. 10% and of the pH-value to 2.5 can not be avoided, but they should not present any difficulties.

The application of the germination test in the routine examination of foods for their preservative content should ensure a fast, reliable, simple and economical operation.

7. Summary.

A germination test for the non-specific detection of preservatives in foods is described,which yields reliable results with a low expenditure of material, time and labor. As a criterion is used the prevention of the germination of conidia spores of A. orycae. Because of its simplicity, the test is particularly suitable for routine examinations.

References.

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

Literatur

[1] v. Schelhorn, M., Zbl. Bakteriol., Parasitenkunde Infektionskrankh., Abt. II, 107, 350 (1952–1954).
[2] Mossel, D. A. A., Food Manufact. 31, 190 (1956).
[3] Coretti, K., Jahresbericht Bundesforsch.anst. Fleischwirtschaft Kulmbach 1955, 29.
[4] Mossel, D. A. A., und de Bruin, A. S., Analyst 78, 37–42 (1953); ref. Z. Lebensmittel-Unters. u. -Forsch. 99, 163 (1954).
[5] Mossel, D. A. A., Mandersloot, J. G., und Gentis, A. S. S., Netherl. Milk Dairy J. 9, 63 (1955); ref. Z. Lebensmittel-Unters. u. -Forsch. 107, 80 (1958).
[6] Koch, J., und Schiffner, G., Die Fruchtsaftindustrie 4, 3 (1959).
[7] Mossel, D. A. A., Zwart, H., und de Bruin, A. S., Analyst 85, 410 (1960).
[8] Bernarts, M. J., Z. Lebensmittel-Unters. u. -Forsch. 104, 405 (1956).
[9] Sandi, E., und Szantha, J., Elelmiszervizsgalati Közlemenyek 6, 141 (1960).
[10] Del Campo, A., Ind. ital. Conserve 32, 288 (1957).
[11] Baum, F. und Lamm, H., Ernährungsforschung 4, 84 (1959).
[12] Baible, K., Z. Lebensmittel-Unters. u. -Forsch. 107, 501 (1958).
[13] Rehm, H. J., Z. Lebensmittel-Unters. u. -Forsch. 110, 375 (1959).
[14] Sommer, L., und Halbsguth, W., Forschungsberichte des Wirtschafts- und Verkehrsministeriums Nordrhein-Westfalen, Nr. 411, 1957.
[15] Haehn, M., Biochemie der Gärungen. Verlag Walter de Gruyter & Co., Berlin 1952, S. 434.
[16] v. Schelhorn, M., Z. Lebensmittel-Unters. u. -Forsch. 92, 256 (1951).
[17] Kahlfeld, F., Nährbodentechnik. Verlag Georg Thieme, Leipzig 1948, S. 59.

Figure 1

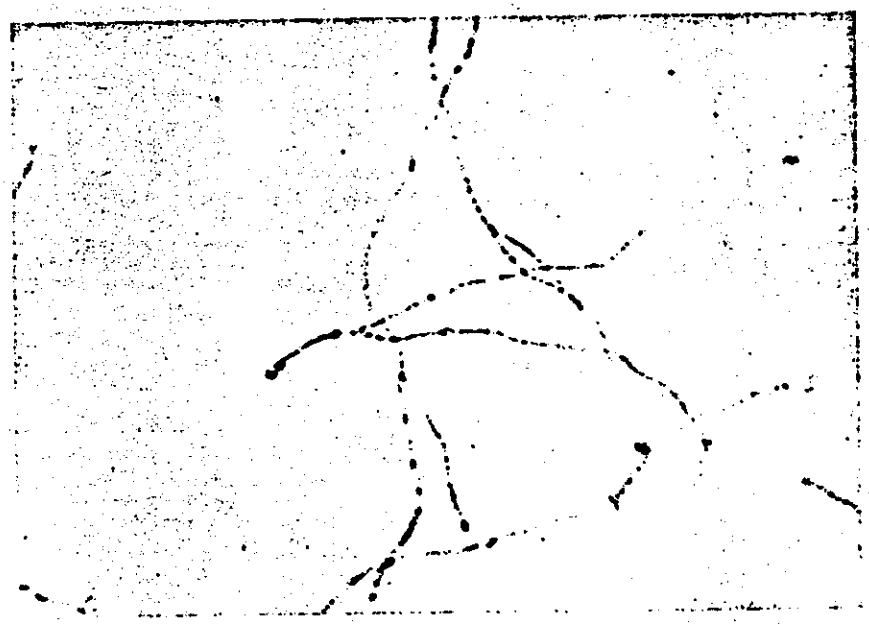

Figure 2

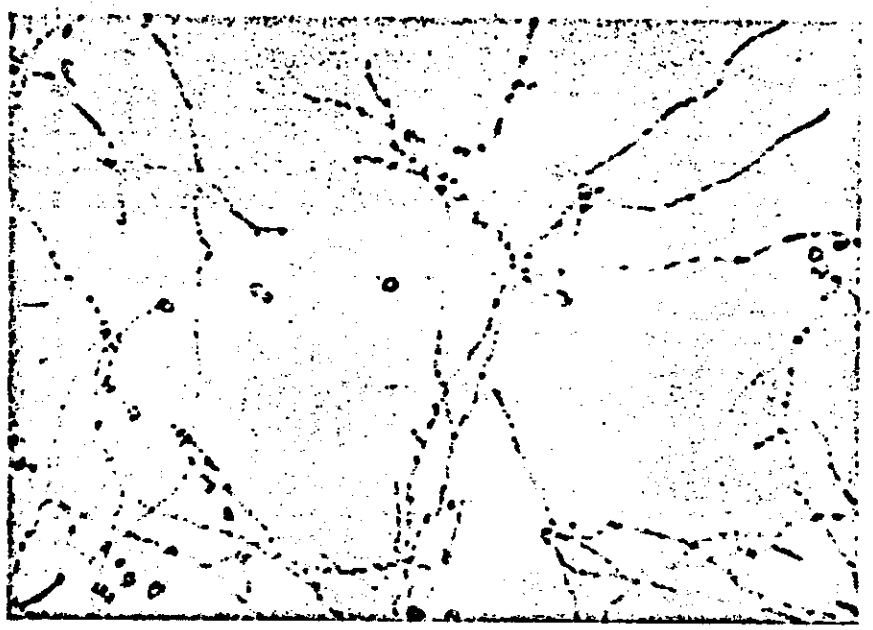

Figure 3

Figure 4

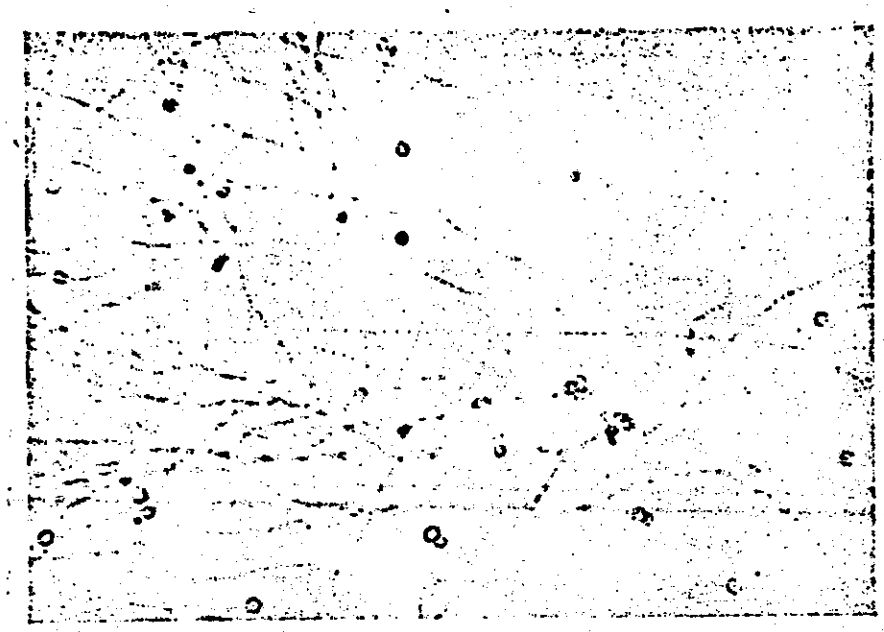

Figure 5

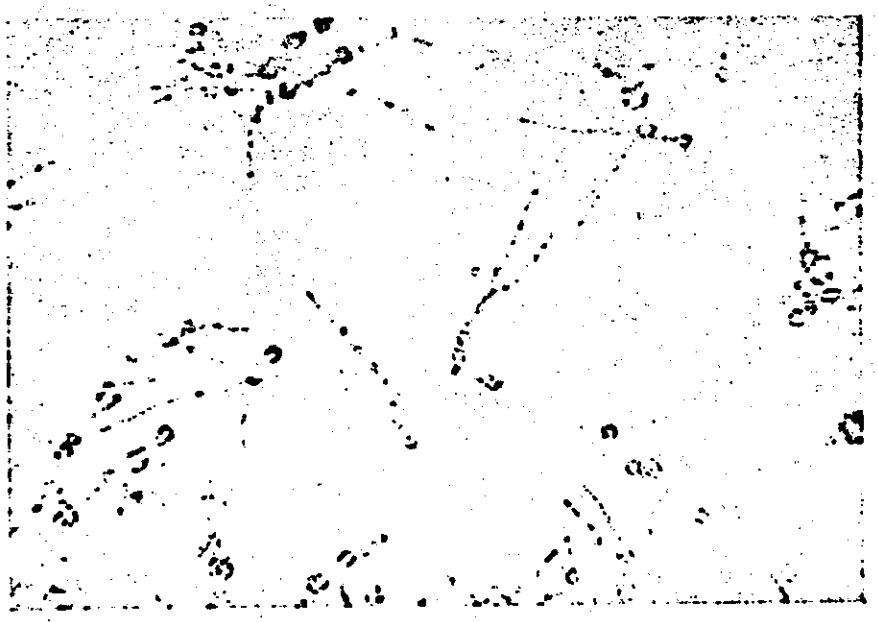

Figure 6

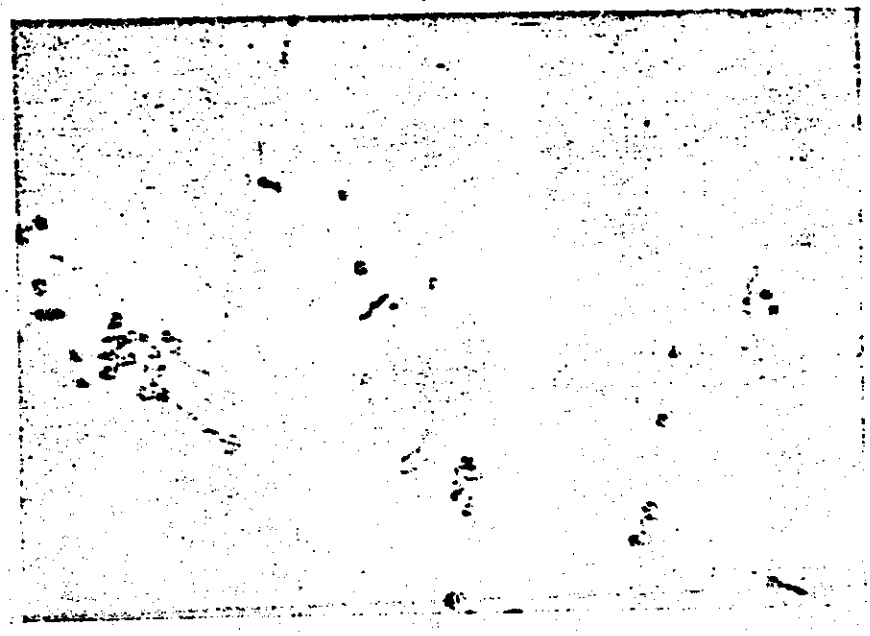

Figure 7

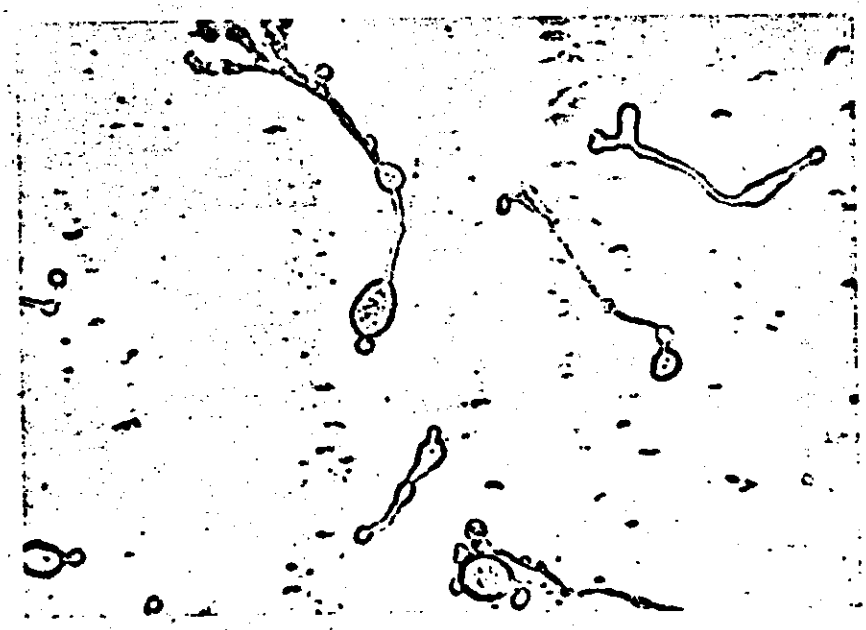

Figure 8

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Ein Keimungstest zum unspezifischen Nachweis von Konservierungsmitteln

von F. Baum und Chr. Wardsack

1. Einleitung

Die Verwendung von Konservierungsmitteln zur Haltbarmachung von Nahrungsmitteln ist in den einzelnen Staaten gesetzlich geregelt. Diese Regelung bezieht sich auf die Art des Konservierungs-Stoffes, die zulässige Höchstzugabe und das zur Konservierung freigegebene Nahrungsmittel. Sinn und Zweck der Zugabe chemischer Konservierungsmittel ist es, Nahrungsmittel durch diesen Zusatz haltbar zu machen, d. h. die enthaltenen Mikroorganismen zumindest eine Zeit lang an ihrer Vermehrung zu hindern. Aus Untersuchungen von v. Schelhorn [1] über die Wirkung von Salicylsäure, Benzoesäure, Ameisensäure und schwefliger Säure auf Willia anomala geht hervor, daß eine dauernde Vermehrungshemmung nicht besteht, sondern daß entweder eine Abtötung oder eine Vermehrung der enthaltenen Mikroorganismen erfolgt. Dabei kann es vorkommen, daß bei niedrigen Konzentrationen des zugegebenen Konservierungsmittels eine Vermehrung erst nach Wochen eintritt. Die Ursachen werden in Resistenzmutationen oder im Abbau des Konservierungsmittels vermutet. Diese von v. Schelhorn [1] gefundenen Ergebnisse entsprechen den immer wieder zu machenden Erfahrungen, die man bei der Lagerung konservierter Nahrungsmittel erhält. In den meisten Fällen setzt ein Verderben der Nahrungsmittel nach Wochen oder Monaten ein.

Diese Erkenntnis über die Wirkung der Konservierungsmittel birgt auch die Möglichkeit in sich, bei der Konservierung von Nahrungsmitteln die gesetzliche Grundlage zu verlassen, um entweder die erlaubte Höchstdosis zu überschreiten oder gar verbotene Mittel zur Konservierung einzusetzen. Diese „illegalen" Konservierungsmittel sind mehr oder weniger giftig, und es befinden sich unter diesen sogar stark giftige Verbindungen wie Derivate der Bromessigsäure, komplexe Quecksilberverbindungen und andere, wie Mossel [2] berichtet. Außerdem ist es möglich, daß aus Unachtsamkeit bei der Konservierung erhöhte Dosen von Konservierungsmitteln in das Nahrungsmittel gelangen. Schließlich kann sich noch die

Möglichkeit einer ungesetzlichen Konservierung aus dem zwischenstaatlichen Handel ergeben, da die Konservierungsmittel-Gesetzgebung der einzelnen Länder verschieden ist.

Bereits aus diesen angeführten Gründen besteht die Notwendigkeit, daß die Nahrungsmittel einer Kontrolle unterzogen werden, um den Verbraucher vor gesundheitlichen Schäden zu bewahren. Bei der großen Mannigfaltigkeit der Verbindungen, die einen konservierenden Effekt ausüben können, ist es sehr schwierig, ein unbekanntes vorhandenes Mittel mit chemischen Methoden zu suchen. Diese Aufgabe wäre äußerst kompliziert und umständlich und auch durch Verwendung der Methoden der Infrarotspektrophotometrie und Chromatographie nicht zu lösen. Es bleibt nur der mikrobielle Test. Dieser beruht darauf, daß bestimmte Mikroorganismen, die man mit einem Nahrungmittel bzw. einem aus diesem bereiteten Extrakt zusammenbringt, normales Wachstum bzw. normale Entwicklung oder typische physiologische Reaktionen zeigen, wogegen eine Hemmung eintritt, wenn das Nahrungsmittel wirksame Mengen an Konservierungsstoffen enthält. Es handelt sich beim mikrobiellen Test um eine unspezifische Methode, mit der sich nur feststellen läßt, ob eine wirksame Komponente vorliegt oder nicht. Für Voruntersuchungen und Routineverfahren sind die mikrobiellen Testmethoden ausgezeichnet geeignet. In der Literatur sind bisher eine große Zahl von Arbeiten über Methoden des unspezifischen Konservierungsmittelnachweises in Nahrungsmitteln erschienen. Es ist hier nicht die Aufgabe, über alle diese Methoden zu diskutieren, ihre Vor- und Nachteile abzuwägen. Der Interessierte findet einen umfassenden Überblick über die verschiedenen Verwendungsmöglichkeiten mikrobieller Methoden in der Arbeit von Mossel [2]. Hier befindet sich auch eine umfangreiche Literaturzusammenstellung.

Der Gärungstest nach Kluyver scheint die gebräuchlichste Nachweismethode zu sein. Verschiedene Modifikationen des Testes wurden z. B. von Mossel [2], Coretti [3], Mossel und de Bruin [4] und Mossel, Mandersloot und Gentis [5] angegeben und beziehen sich z. T. auf die Extraktion der wirksamen Stoffe, wenn das Nahrungsmittel nicht selbst als Medium dienen kann, so z. B. Fleisch. Der Kluyver-Test, der auf gasometrischer Technik beruht, basiert auf dem Prinzip, daß das zu testende Nahrungsmittel im einfachsten Falle nach Zugabe bestimmter Mengen an Nährstoffen und Hefezellen direkt vergoren wird. Die Reaktion wird nach 24 Stunden in Abständen von 3 Stunden abgelesen, indem die durch die Gärung der Hefe aus Dextrose entstehende CO_2-Menge gemessen wird. Die Reaktion gilt als beendet, wenn sich in einem konservierungsmittelfreien Extrakt die maximale Gasmenge gebildet hat. Man schließt auf die Gegenwart von Konservierungsmitteln, wenn nach 48 Stunden keine Gärung eingetreten ist. Verdacht auf Konservierungsmittelgehalt besteht, wenn nach 33 Stunden keine oder eine verzögerte Gasbildung vorhanden ist, aber nach 48 Stunden maximale Vergärung eintritt.

Eine ähnliche, auf dem Gärtest basierende Nachweismethode beschreiben Koch und Schiffner [6] für den unspezifischen Nachweis von chemischen Konservierungsmitteln in Fruchtsäften (Süßmosten), alkoholfreien Erfrischungsgetränken und Obstkonzentraten. Allerdings ergibt sich hier eine gewisse methodische Schwierigkeit. So machen sich 100 mg% p-Oxybenzoesäure nach 15stündiger Bebrütung als Gärverzögerung bemerkbar; nach 24 Stunden soll die Gärung wieder normal verlaufen. Recht merkwürdig mutet der ebenfalls erwähnte „Schimmelpilztest" an. Hierbei werden je 25 ml Most in sterilisierte Petrischalen gegeben und die Schalen ohne Deckel einer 60 Minuten langen „Zufallsinfektion" durch Zimmerluft ausgesetzt. Nach einer 3tägigen Bebrütung bei 25 °C schließt man bei der Auswertung von gewachsenen Schimmelpilzkulturen auf konservierungsmittelfreien Most. Mit dieser Methode läßt sich jedoch nicht einmal eine positive Infektion, abgesehen von der Art der Schimmelpilze, nachweisen. Ein anderer Test fußt auf dem Wachstum von Candida brumptii und ist beschrieben von Mossel, Zwart und de Bruin [7]. Von der Art des Wachstums der Hefezellen auf Agarplatten, die das zu untersuchende Nahrungsmittel homogenisiert enthalten, schließt man auf Gegenwart oder Fehlen von Konservierungsmitteln.

Bernarts [8] verwendet die osmophile Hefe Saccharomyces rouxii zum unspezifischen Nachweis von Konservierungsmitteln in Maemelade. Sandi und Szantha [9] arbeiten nach dem Kluyver-Mossel'schen Verfahren mit Saccharomyces cerevisiae, aber mit verändertem Fermentierungsrohr, das sich für systematische Serienuntersuchungen eignen soll. Del Campo [10] wiegt die beim Gärtest mit Saccharomyces cerevisiae entstehende CO_2-Menge.

Nach dieser kurzen Aufzählung nur einiger Methoden soll noch die Verwendbarkeit im Routineverfahren erwähnt werden. Nach unserer Meinung sollte eine Test-Methode, die sich für Serienuntersuchungen eignen soll, folgende Bedingungen erfüllen:

1. Der Test muß einfach durchführbar sein, d. h. ohne komplizierte technische und optische Apparate.
2. Spätestens am folgenden Tag, d. h. nach 24 Stunden, muß das Untersuchungsergebnis vorliegen.
3. Das Bearbeiten der Probe darf nicht viel Zeit erfordern.
4. Es müssen bereits kleine Mengen an Konservierungsmitteln nachweisbar sein.
5. Auch in Nahrungsmittelresten, z. B. 1—2 ml eines flüssigen Nahrungsmittels, müssen die evtl. enthaltenen Konservierungsmittel noch sicher nachgewiesen werden können.
6. Die verwendeten Glasgeräte müssen leicht zu reinigen sein.
7. Die notwendigen mikrobiologischen Arbeiten müssen auch von Mitarbeitern durchgeführt werden können, die auf diesem Gebiet nicht geschult sind.

Wenn man diese Gesichtspunkte auf die oben erwähnten Methoden bezieht, muß folgendes festgestellt werden: der Kluyver'sche Test, verschiedentlich modi-

liziert, dauert bei einer Versuchszeit von 48 Stunden zu lange. Außerdem sind die verwendeten Gärungssaccharometer nach Einhorn nur äußerst umständlich zu reinigen (es ist möglich, daß die von CORETTI [3] beschriebenen Gärungsprouvetten, die von uns nicht verwendet wurden, leichter zu säubern sind). Über unsere Erfahrungen bezüglich der Genauigkeit dieses Testes berichteten wir bereits kurz (BAUM und LAMM [11]). Es fällt teilweise nicht leicht, positive und negative Resultate eindeutig zu trennen, da in Parallelansätzen z. T. starke Streuwerte auftreten. Die von KOCH und SCHIFFNER [6] beschriebene Methode besitzt außer den eben genannten Nachteilen noch die Schwierigkeit einer Ablesung nach 15 Stunden. Das läßt sich mit einer 8stündigen bzw. $7^1/_2$stündigen Arbeitszeit der Mitarbeiter schlecht vereinbaren. Der „Schimmelpilztest" liefert mehr oder weniger Zufallsergebnisse und scheidet deshalb aus. Unsere Forderung, auch in kleinsten Nahrungsmittelresten noch Konservierungsmittel nachweisen zu können, wird von keiner der genannten Methoden erfüllt. Der Agar-Diffusionstest, der von uns (BAUM und LAMM [11]) für den Nachweis von Konservierungsmitteln eingesetzt werden sollte, ist für Routinearbeiten ungeeignet und besitzt praktisch nur noch theoretisch Interesse.

Bei der Ausarbeitung einer Methode, die den Belastungen von Serienuntersuchungen standhalten muß, gingen wir auch davon aus, daß dieser Test mit nur geringem Arbeitsaufwand durchgeführt werden darf. Auch die Rentabilität spielt eine gewisse Rolle. Als Hauptgesichtspunkte betrachten wir jedoch die oben von uns aufgestellten 7 Bedingungen.

2. Die Durchführung des Keimungstestes

Den Anstoß zur Ausarbeitung eines Keimungstestes gaben neben eigenen Überlegungen Arbeiten von RAIBLE [12] und REHM [13]. Raible schreibt über: „Ein einfaches Verfahren zur Prüfung der Wirksamkeit chemischer Konservierungsmittel gegen Schimmelpilze", und Rehm teilt seine Erfahrungen bei der Anwendung dieser Methode mit. Die Durchführung dieses Testes ist folgendermaßen:

Filtrierpapierscheiben werden mit den verschiedenen Verdünnungsstufen des Hemmstoffes in Nährmedium getränkt und mit Sporen bzw. Hyphenmaterial beimpft in einer Petrischale (feuchte Kammer) ausgelegt. Die verwendeten Organismen waren Aspergillus niger und Fusarium nivale. Die Entwicklungsdauer der Pilze bei der Bebrütung betrug 3—4 Tage. Nach dieser Zeit erfolgte die Auswertung der Proben durch Ablesen der Myceleutwicklung oder durch Jodprobe. Als Vorteile der Methode werden Platz- und Materialersparnis und höhere Genauigkeit genannt. Will man diese Methode zum Nachweis von Konservierungsmitteln in Nahrungsmitteln benutzen, so kommt als Nachteil die lange Bebrütungszeit in Betracht.

Wir gingen davon aus, daß man als Kriterium nicht das Mycelwachstum betrachtet, sondern die erfolgte Keimung der Pilzsporen. Da die Keimung unter bestimmten Bedingungen innerhalb 24 Stunden erfolgt, läßt sich die Versuchszeit bis zur Auswertung erheblich verkürzen.

Zur Beobachtung der Keimung von Pilzsporen ist es üblich, die Kultur im sogenannten „hängenden Tropfen" durchzuführen. Diese Methode ist selbstverständlich nicht für eine Routine-Untersuchung geeignet. Wir arbeiteten deshalb ohne Deckglas und pipettierten 0,05 ml der Versuchslösungen in den Hohlschliff der Objektträger und beimpften mit etwas Sporenmaterial des Schimmelpilzes Aspergillus orycae. Die so präparierten Objektträger wurden in große Petrischalen verbracht, in denen sich Rundfilter befinden, die mit 6%iger NaCl-Lösung getränkt sind. Da die Versuchslösungen auf einen Zuckergehalt von ca. 10% eingestellt sind, erhalten wir nach dem Bedecken der Schalen eine feuchte Kammer. Es kommt auf diese Weise weder zu einem Austrocknen der Tropfen, noch zu einer Wasseranziehung, wodurch verständlicherweise die Konservierungsmittelkonzentration gleich bleibt. Die so angesetzten Versuche werden 24 Stunden bei +30 °C bebrütet und nach dieser Zeit ausgewertet, indem die im Hohlschliff der Objektträger befindlichen Tropfen einer mikroskopischen Kontrolle bei schwacher Vergrößerung unterzogen werden. Die erfolgte Keimung ist an der Bildung von Keimschläuchen zu erkennen. In den meisten Fällen ist eine mikroskopische Durchsicht nicht notwendig, da man schon mit dem unbewaffneten Auge ein gewachsenes dichtes Mycel erkennen kann, wenn der Test negativ ausfällt. Sommer und Halbsguth [14] deuten bei Phycomyces-Sporangiosporen bereits eine im Sporeninnern auftretende Vakuolenbildung als erfolgte Keimung. Dieses Kriterium ist aber im schnell arbeitenden Routine-Betrieb nicht zu verwenden, so daß wir hier die Ausbildung von Keimschläuchen als Kriterium ansehen. Diese Keimschläuche haben oftmals, in Abhängigkeit vom verwendeten Konservierungsmittel, unterschiedliches Aussehen (S. Abb. 1--8).

Es kann vorkommen, daß nicht alle Sporen, bzw. nur wenige Konidiosporen ausgekeimt sind. Wir haben eine über 50% liegende Prozentzahl an gekeimten Sporen als positiv gewertet. Sind nur einzelne Sporen ausgekeimt, so wird dies als negativ oder „nicht gekeimt" bewertet. Die Kontrolle der Versuchsansätze verläuft außerordentlich schnell, da ein kurzer Überblick genügt, um eine positive Keimung festzustellen. Es braucht nichts gemessen oder gezählt zu werden. Eine nach 24 Stunden Bebrütungszeit sichtbare Keimung wird gedeutet als „kein Konservierungsmittel in der Lösung" oder „keine wirksame Menge an Konservierungsmittel in der Lösung". Hat die Keimung der Sporen innerhalb 24 Stunden nicht begonnen, so wird das als „wirksame Menge an Konservierungsmittel in der Lösung" bewertet. Die zum Versuch verwendete Nährlösung, modifiziert nach Haehn [15], hat folgende Zusammensetzung:

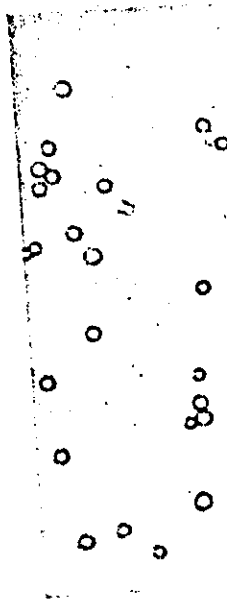


Abb. 1. Ungekeimte

Abb. 2. Ausgek

icht das Mycelwachstum be-
ren. Da die Keimung unter
igt, läßt sich die Versuchszeit

t es üblich, die Kultur im
Diese Methode ist selbst-
ignet. Wir arbeiteten deshalb
slösungen in den Hohlschliff
material des Schimmelpilzes
wurden in große Petrischalen
%iger NaCl-Lösung getränkt
alt von ca. 10 % eingestellt
ı eine feuchte Kammer. Es
ı der Tropfen, noch zu einer
Konservierungsmittelkonzen-
den 24 Stunden bei +30 °C
e im Hohlschliff der Objekt-
ontrolle bei schwacher Ver-
st an der Bildung von Keim-
e mikroskopische Durchsicht
eten ge ein gewachsenes
ativ ausfällt. SOMMER und
oren bereits eine im Sporen-
mung. Dieses Kriterium ist
verwenden, so daß wir hier
sehen. Diese Keimschläuche
Konservierungsmittel. unter-

nur wenige Konidiosporen
Prozentzahl an gekeimten
ausgekeimt, so wird dies als
ter Versuchsansätze verläuft
ı, um eine positive Keimung
ıhlt zu werden. Eine nach
gedeutet als „kein Konser-
Menge an Konservierungs-
innerhalb 24 Stunden nicht
onservierungsmittel in der
ıhrlösung, modifiziert nach

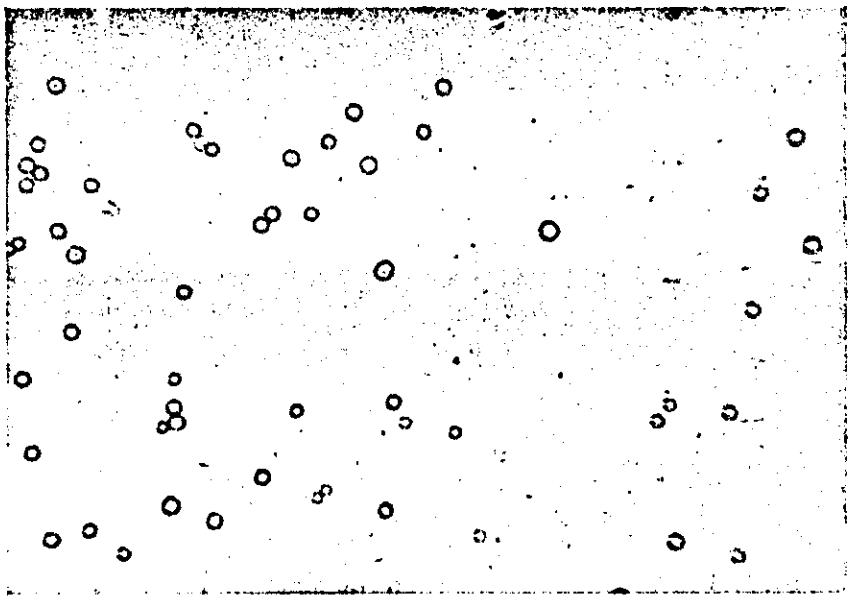


Abb. 1. Ungekeimte Konidiosporen von Aspergillus orycae. Vergrößerung: ca. 300fach.

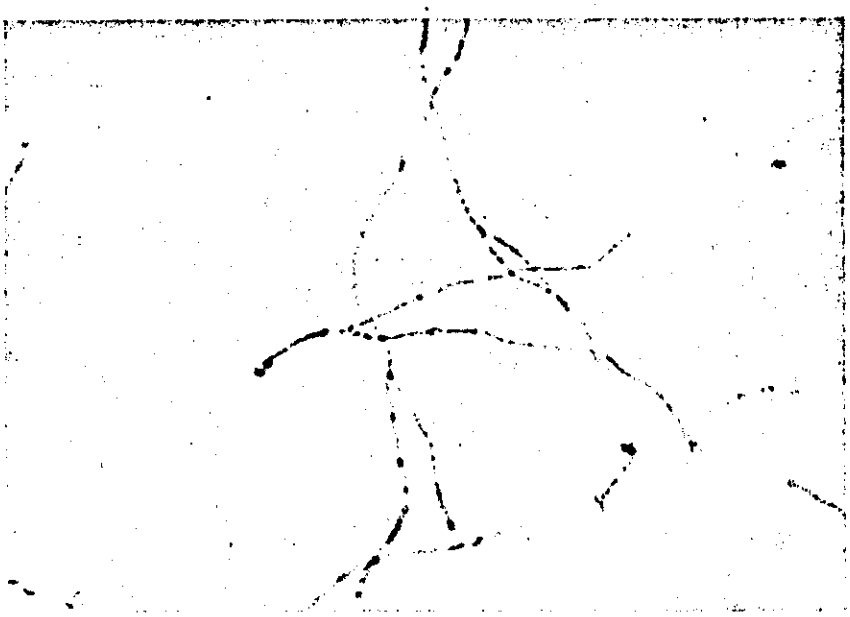


Abb. 2. Ausgekeimte Konidiosporen von Aspergillus orycae. Konservierungsmittel: Na-Sorbinat 25 mg%; Vergrößerung: ca. 300fach

Tafel 2

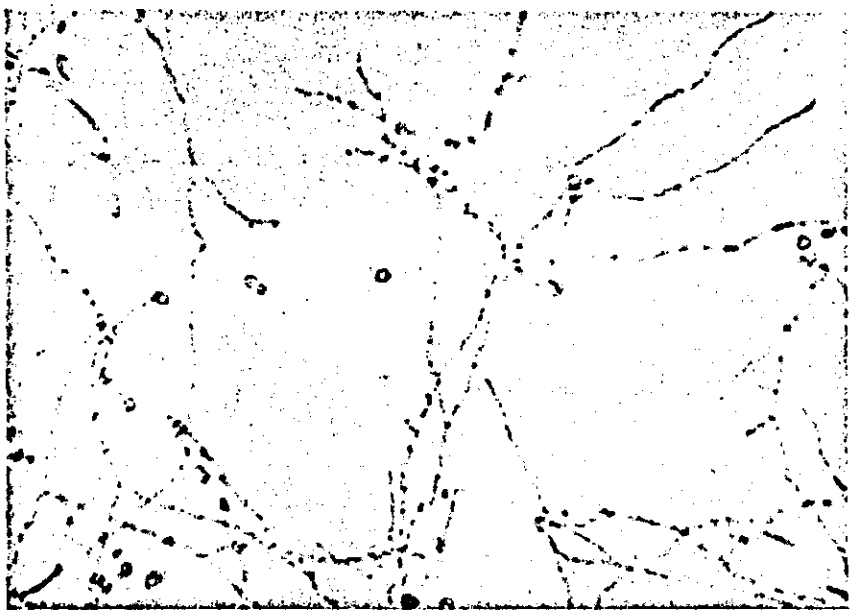


Abb. 3. Ausgekeimte Konidiosporen von Aspergillus orycae. Konservierungsmittel: Sorbinsäure 5 mg%; Vergrößerung: ca. 300fach

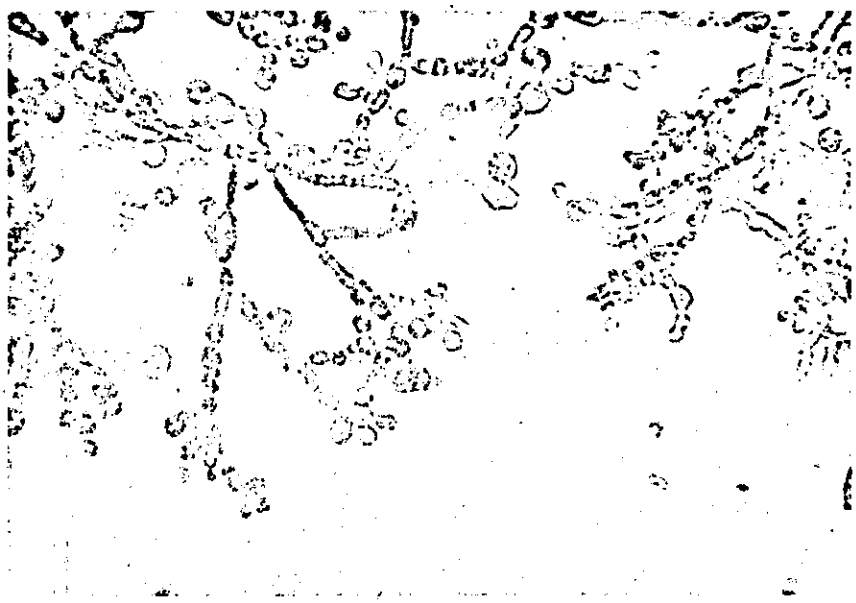


Abb. 4. Ausgekeimte Konidiosporen von Aspergillus orycae. Konservierungsmittel: Nipagin A 10 mg%; Vergrößerung: ca. 300fach

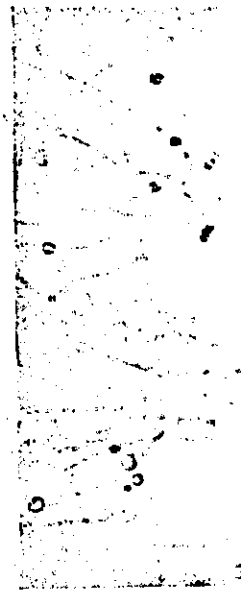


Abb. 5. Ausgekeimte K
Na-P

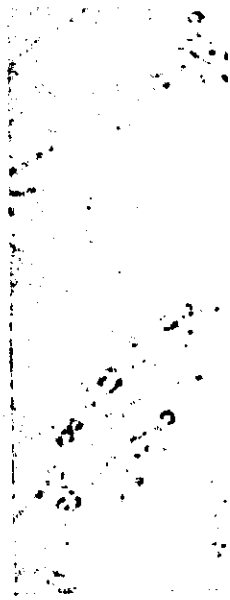


Abb. 6. Ausgekeimte
N

Konservierungsmittel:
ach

Konservierungsmittel:
ach

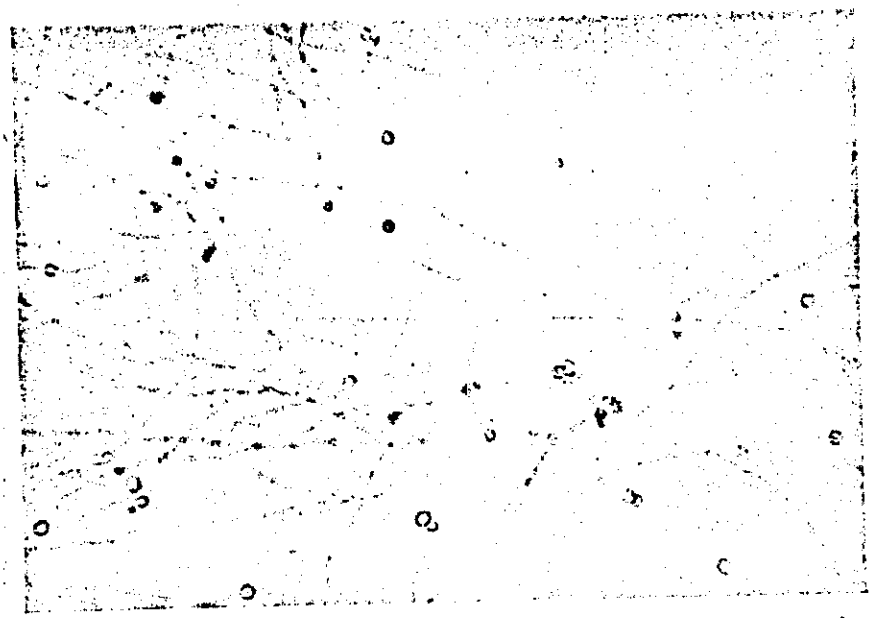


Abb. 5. Ausgekeimte Konidiosporen von Aspergillus oryzae. Konservierungsmittel: Na-Benzoat 2,5 mg%; Vergrößerung: ca. 300fach

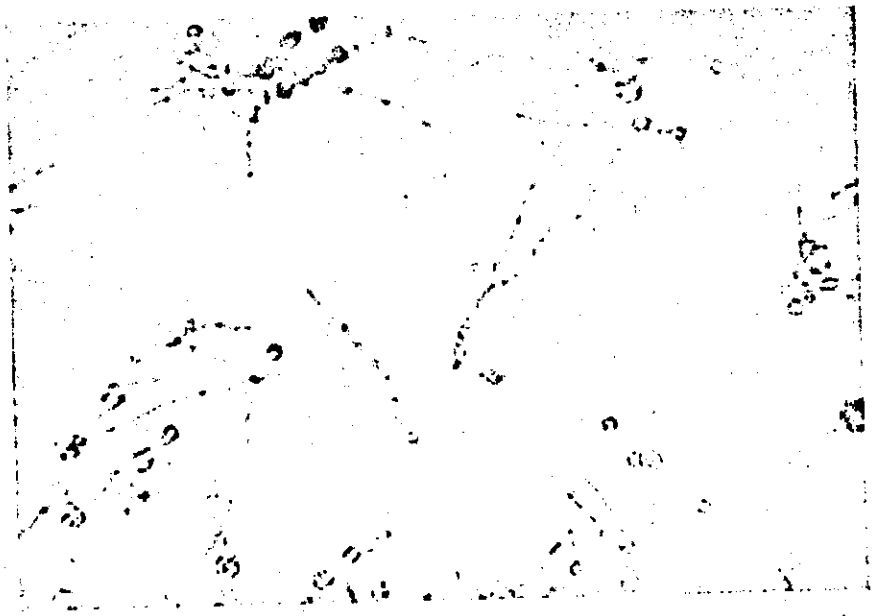


Abb. 6. Ausgekeimte Konidiosporen von Aspergillus oryzae. Konservierungsmittel: Nipasol 5 mg%; Vergrößerung: ca. 300fach

Tafel 4

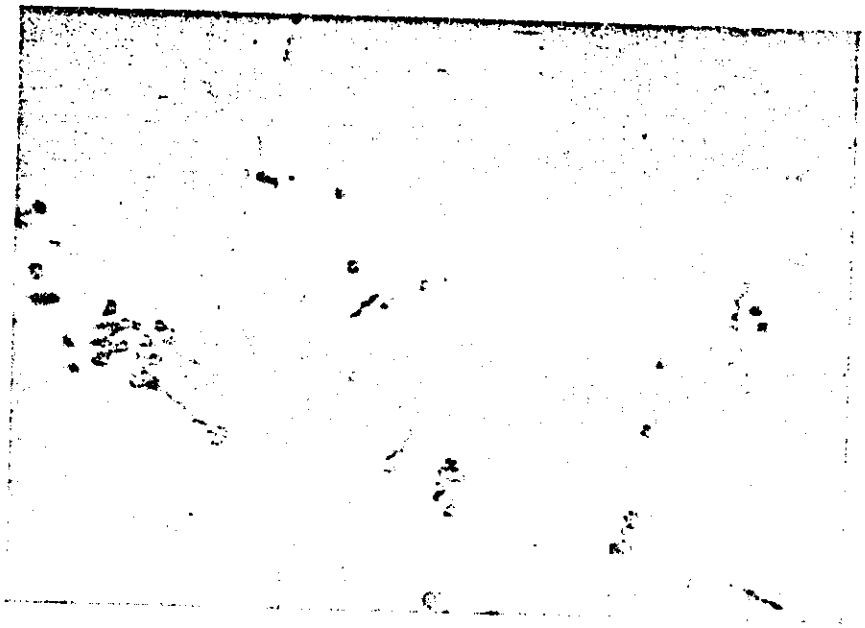


Abb. 7. Beginn der Keimung von Konidiosporen von Aspergillus oryzae. Konservierungsmittel: Benzoesäure 5 mg%; Vergrößerung: ca. 300fach

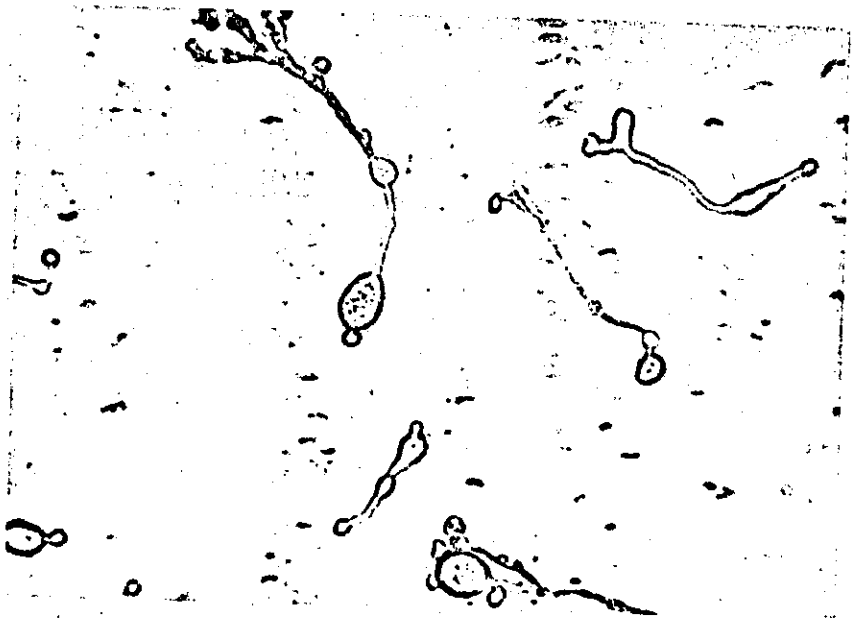


Abb. 8. Ausgekeimte Konidiosporen von Aspergillus oryzae. Kontrolle ohne Konservierungsmittel; Vergrößerung: ca. 300fach

2,5 g NH_4NO_3
1,0 g KH_2PO_4 pH = 2,5
0,25 g $MgSO_4$
100,0 g Saccharose
ad 1000 ml aqua dest.

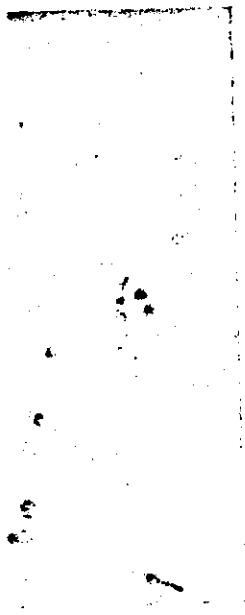
Die Lösung wird im Koch'schen Dampftopf 30 Minuten lang sterilisiert. In dieser Nährlösung werden die verschiedenen Konservierungsmittel gelöst und entsprechende Verdünnungsreihen hergestellt. Von besonderer Wichtigkeit ist, daß jeweils der pH-Wert auf 2,5 ± 0,1 eingestellt wird. Dieser niedrige pH-Wert wurde absichtlich gewählt, da nach v. SCHELHORN [16] die wirksame Komponente im Konservierungsmittel das undissoziierte Molekül ist und der undissoziierte Anteil der meisten Konservierungsmittel ja mit fallendem pH-Wert steigt. 0,05 ml der Nährlösung mit oder ohne Konservierungsmittel werden, wie bereits beschrieben, auf die Hohlschliffobjektträger aufgetragen. Man setzt jeweils 3 Parallelproben an und beimpft mit wenig Sporen von Aspergillus orycae. Diese Sporen nimmt man mit einer Stich-Nadel von einer Kulturplatte des Schimmelpilzes ab und bringt sie in den im Hohlschliff befindlichen Tropfen Untersuchungslösung. Der Schimmelpilz Aspergillus orycae wird auf Grütz-Agar [17] folgender Zusammensetzung gezogen:

Biomalz	60 g
Pepton	5 g
NaCl	5 g
Glycerin	5 ml
Agar	30 g
Nährbouillon	1 l

Der Nährboden mit dem pH 7,2 wird im Dampftopf kurz aufgekocht und anschließend filtriert. Die Sterilisationszeit beträgt 30 Minuten bei 121 °C im Autoklaven.

Die mit Aspergillus orycae beimpften Platten werden bei 30 °C bebrütet. Die beimpften Versuchsansätze werden, wie bereits beschrieben, in Petrischalen verbracht, in denen sich ein mit einer 6% NaCl-Lösung getränktes Filtrierpapier befindet, und bei 30 °C 24 Stunden lang bebrütet. Anschließend Kontrolle der Objektträger. Es muß betont werden, daß Petrischalen, Filtrierpapier sowie die Hohlschliffobjektträger nicht steril zu sein brauchen, so daß dieser Arbeitsgang bei Anwendung des Keimungstestes entfällt; auch die Nährlösung braucht nicht sterilisiert zu werden. Wir haben die Sterilisation der Nährlösung nur durchgeführt, um diese über eine längere Zeit haltbar zu machen.

Es soll an dieser Stelle erwähnt werden, daß die Züchtung des Schimmelpilzes auch für einen ungeübten Mitarbeiter sehr einfach ist. Um den Test weiter zu



ilus ae. Konservierungs-
a. 300fach

Kontrolle ohne Konservierungs-
h

vereinfachen, sind wir dazu übergegangen, mit Sporenkonserven zu arbeiten, so daß die Züchtung des Testorganismus vollkommen entfallen dürfte. Entsprechende Versuche sind fast abgeschlossen, und es wird später darüber berichtet.

3. Über die Verwendung von Aspergillus oryzae als Testorganismus

Erste Versuche unter Verwendung von Aspergillus-niger-Konidiosporen brachten keine befriedigenden Ergebnisse. Die Ursache hierfür dürfte darin zu suchen sein, daß Aspergillus niger, zumindest die von uns verwendeten Stämme, noch bei Gegenwart hoher Dosen an Konservierungsmitteln auskeimen, also verhältnismäßig resistent sind, bzw. in der Lage sind, Konservierungsmittel abzubauen. Aspergillus oryzae als Testorganismus spricht bereits auf geringe Mengen von Konservierungsmittel an und hat sich bei der Bestimmung bei nunmehr fast 3jährigem Einsatz sehr gut bewährt.

4. Ergebnisse der Methode

Verschiedene chemische Konservierungsmittel wurden bisher mit dem Keimungstest geprüft. Zwecks Feststellung der Grenzhemmkonzentration wurden die Mittel in mehreren Konzentrationen ausgetestet. Es handelt sich bei den Substanzen sowohl um erlaubte als auch um verbotene Konservierungsmittel. Folgende Stoffe wurden bisher untersucht:

Sorbinsäure	Dehydracetsäure
Natrium-Sorbinat	Hexamethylentetramin
Benzoesäure	Ameisensäure
Natrium-Benzoat	Nipasol
Salicylsäure	Nipagin A
Natrium-Salicylat	Natriumnitrit
Borsäure	Kochsalz.

Die schwer wasserlöslichen Säuren und Ester wurden zwecks besseren Lösens im Wasserbad bei +50 °C behandelt.

Versuche mit Nährlösung ohne Konservierungsmittel ergaben, daß in diesem Medium die Keimung der Sporen nach 10stündiger Bebrütung bei 30 °C bereits erfolgt war. Die Festlegung der Bebrütungszeit auf 24 Stunden erfolgte willkürlich. Diese Zeit erschien uns beim Routine-Verfahren als die günstigste, da die Auswertung dann zur gleichen Tageszeit erfolgt, so daß Verwechslungen der Proben fast ausgeschlossen sind.

Folgende Grenzhemmkonzentrationen wurden mit dem Keimungstest erhalten:

Nachweisbare Mengen an Konservierungsmitteln (Keimungshemmung):

Sorbinsäure	10 mg %
Na-Sorbinat	30—50 mg %
Benzoesäure	10 mg %
Na-Benzoat	10 mg %
Salicylsäure	1,0 mg %
Na-Salicylat	2,5 mg %
Borsäure	750—1000 mg %
Dehydracetsäure	10—25 mg %
Hexamethylentetramin	200—250 mg %
Ameisensäure	200—250 mg %
Nipasol	10 mg %
Nipagin A	15—20 mg %

Wertet man bereits den negativen Ausfall der Einzelproben von Parallelansätzen (es besteht eine gewisse Übergangsbreite von „gekeimt" zu „nicht gekeimt", die sich bemerkbar macht durch die Keimung von etwa 50% der eingesäten Sporen oder „+" und „—" der Einzelproben), so erhält man folgende Ergebnisse:

Nachweisbare Mengen an Konservierungsmitteln:

Benzoesäure	2,5 — 5,0 mg %	Dehydracetsäure	5,0 —10,0 mg %
Na-Benzoat	5,0 —10,0 mg %	Nipasol	2,5 — 5,0 mg %
Salicylsäure	0,25— 0,5 mg %	Nipagin A	5,0 —10,0 mg %

Die Gegenwart von Kochsalz bis zu 5 g% sowie 1 g% Natriumnitrit stört den Test nicht.

Vergleicht man die Werte mit den Ergebnissen von Koch und Schiffner [6] und Coretti [3], die beide mit dem Gärtest arbeiten, so ergibt sich folgende Gegenüberstellung:

Nachweisbare Mengen an Konservierungsmitteln in mg %

Konservierungsmittel	Keimungstest	nach Coretti	nach Koch und Schiffner
Benzoesäure	10	25—50	10—30
Natriumbenzoat	10	25—50	15—20
Nipasol	10	×	25—75
Nipagin A	15—20	100—500	40—120
Ameisensäure	200—250	50—100	150—200
Dehydracetsäure	10—25	×	25—50
Sorbinsäure	10	×	25—50
Natriumsalicylat	2,5	25	×
Borsäure	750—1000	100—200	×

× = keine Angaben.

nungstest mit Ausnahme
fittel in bereits geringeren
uch die Berücksichtigung
chinkenextrakt gelöst hat
erhältnissen nichts; denn,
en wir z. B. bei Apfelsaft
gefunden.

Zeit und Sporenalter

: tiefen pH-Wert zu arbei-
m Test auszunutzen. Nach
ngsmittel weitgehend ab-
onservierungsmittel. Alle
Reaktion weitgehend un-
wird der Schluß gezogen.
ervierende Wirkung ver-

oll hier angeführt werden.
Wi g mit abnehmen-

v. SCHELHORN [16])

er Anteil in % bei		
pH 5	pH 6	pH 7
0,94	0,094	0,0094
4,5	0,47	0,048
9,7	1,06	0,107
13	1,5	0,15
42	6,7	0,71

aß wir mit dem pH-Wert
n. Auch die Verwendung
ist in diesem Zusammen-

e und pH-Papier Stuphan
n 0,3 Einheiten. Es ist
ellt wird, da sonst nicht
e 2 beweist:

Tabelle 2

Nachweisbare Mengen an Konservierungsmitteln in Abhängigkeit vom pH-Wert (Versuchsdauer 24 Std.; Bebrütungstemperatur 30 °C) in mg%

Konservierungsmittel	pH = 2,5	pH = 3,0	pH = 4,0
Sorbinsäure	10	10	25
Na-Sorbinat	30–50	50	>200
Benzoesäure	10	10	25
Na-Benzoat	10	10	100
Salicylsäure	1,0	2,5– 5,0	25
Na-Salicylat	2,5	5,0–10,0	>100

Aus der Tabelle wird deutlich ersichtlich, daß mit steigendem pH-Wert die hemmende Wirkung auf Aspergillus orycae nachläßt. Dabei ist der Unterschied zwischen pH 3,0 und pH 4,0 besonders bemerkenswert.

Die Versuchsdauer wurde, wie oben beschrieben, von uns auf 24 Stunden festgesetzt. Zu diesem Zeitpunkt sind die Sporen der Kontrolle (ohne Konservierungsmittel) bereits gekeimt, und es hat sich ein auch ohne Mikroskop sichtbares, dichtes, weißes Mycel ausgebildet. Um die Frage zu klären, ob die nach 24-stündiger Bebrütung nicht gekeimten Sporen ihre Keimfähigkeit durch Einwirkung der Konservierungsmitteldosis verloren haben oder ob nur eine Verzögerung der Keimung vorliegt, wurden Versuche unter Anwendung verschiedener Konservierungsmittel nach 24stündiger und 48stündiger Bebrütungsdauer ausgewertet. Die Ergebnisse sind in Tabelle 3 aufgeführt:

Tabelle 3

Keimung der Konidiosporen von Aspergillus orycae nach 24- und 48stündiger Bebrütungsdauer bei 30 °C

Konservierungsmittel		24 Std.	48 Std.
Na-Sorbinat	25 mg%	+ + +	
Na-Sorbinat	50 mg%	– – –	+ + ∓
Benzoesäure	5 mg%	+ + +	
Benzoesäure	10 mg%	– – –	+ + +
Benzoesäure	25 mg%	– – –	+ + +
Na-Benzoat	10 mg%	– – –	+ + +
Na-Salicylat	2,5 mg%	– – –	– – –
Salicylsäure	0,5 mg%	+ + –	+
Salicylsäure	1,0 mg%	– – –	– – –

Erklärung: bei jedem Versuch 3 Parallelproben
+ gekeimt
– nicht gekeimt
∓ etwa 50% der Sporen gekeimt

Es hat sich gezeigt, daß sich die von uns angegebenen Grenzhemmkonzentrationen nur auf eine Bebrütungszeit von 24 Stunden beziehen. Diese Zeit ist ebenfalls unbedingt beim Test einzuhalten. Nach 48 Stunden sind auch zum Teil die Sporen gekeimt, die einer höheren Konservierungsmittel-Dosis ausgesetzt sind. Besonders deutlich wird das bei Benzoesäure. Dagegen wird durch Zusatz von 2,5 mg% Na-Salicylat oder 1,0 mg% Salicylsäure auch nach 48stündiger Bebrütungszeit noch die Keimung verhindert. Das läßt jedoch nicht die Aussage zu, daß die Sporen ihre Keimfähigkeit verloren haben, da nach dieser Zeit der Versuch abgebrochen wurde. Es besteht also hier die Möglichkeit einer weiteren Keimungsverzögerung. Die zum Test verwendeten Konidiosporen wurden 3—6 Tage alten Kulturen von Aspergillus orycae abgenommen. Hierbei wurde kein Unterschied in der Keimfähigkeit festgestellt. Versuche mit älteren Sporen werden zur Zeit noch durchgeführt, um, wie bereits erwähnt, die Möglichkeit einer Verwendung von Sporenkonserven zu prüfen.

6. Diskussion

Der beschriebene Keimungstest unter Verwendung von Aspergillus orycae als Testorganismus eignet sich nach unserer Meinung gut für die Serienuntersuchung zum unspezifischen Nachweis von Konservierungsmitteln in Nahrungsmitteln. Der Keimungstest besitzt mehrere Vorteile gegenüber anderen Methoden. So lassen sich in den meisten Fällen bereits geringe Mengen an Konservierungsmitteln nachweisen. Das dürfte vor allem bedingt sein durch den für die Untersuchung günstigen pH-Wert von 2,5, bei welchem die meisten Konservierungsmittel eine bessere Wirkung zeigen als bei pH 4,0, der beim Gärtest zur Anwendung kommt. Ein weiterer Vorteil ist, daß nur kleine Mengen an Nahrungsmitteln für den Test benötigt werden (1 ml eines flüssigen Nahrungsmittels ist vollständig ausreichend), und das Ergebnis der Untersuchung bereits nach 24 Stunden vorliegt. Die Festlegung der Bebrütungsdauer auf 24 Stunden schließt dabei falsche Auswertungszeiten weitgehend aus, da die Auswertung zur gleichen Tageszeit erfolgt wie das Ansetzen der Proben, nur jeweils einen Tag später. Die Ablesung der Ergebnisse erfolgt nur einmal und braucht nicht mehrmals durchgeführt zu werden wie beim Gärtest. Die Auswertung erfolgt bei schwacher Vergrößerung unter dem Mikroskop. Ist kein Konservierungsmittel oder keine wirksame Menge davon enthalten, ist eine mikroskopische Durchsicht nicht notwendig, da das dann gewachsene Mycel bereits ohne mikroskopische Vergrößerung sichtbar ist. Als Vorteil ist auch zu werten, daß die benötigten Glasgeräte (Petrischalen und Hohlschliffobjektträger) billig in der Anschaffung und rentabel im Gebrauch sind und sich leicht reinigen lassen. Sterilisierung der Glasgeräte sowie der Probenlösungen ist überflüssig.

Nachteilig macht sich beim Keimungstest immer noch bemerkbar, daß mikrobiologische Züchtungsarbeiten notwendig sind. Die Anzucht des Schimmelpilzes

Aspergillus orycae ist zwar einfach und bereitet auch mikrobiologisch nicht geschulten Personen keinerlei Schwierigkeiten, hält aber im chemischen Laboratorium oftmals die laufenden Arbeiten auf, da hierbei unter sterilen Bedingungen gearbeitet werden muß. Falls unsere Untersuchungen über die Verwendung von Sporenkonserven einen erfolgreichen Abschluß erreichen, dürfte damit der Keimungstest eine nicht unbeträchtliche Vereinfachung erfahren. Das Einstellen des Untersuchungssubstrates auf einen Zuckergehalt von ca. 10 % sowie des pH-Wertes auf 2,5 ist nicht zu umgehen und dürfte auch keine besonderen Schwierigkeiten bereiten.

Die Anwendung des Keimungstestes für die Routine-Untersuchung von Nahrungsmitteln auf Konservierungsmittelgehalt dürfte ein schnelles, sicheres, einfaches und rentables Arbeiten gewährleisten.

7. Zusammenfassung

Ein Keimungstest zum unspezifischen Nachweis von Konservierungsmitteln in Nahrungsmitteln wird beschrieben, der bei nur geringem Aufwand an Material, Zeit und Arbeitskräften sichere Ergebnisse liefert. Als Kriterium dient dabei die Verhinderung der Keimung der Konidiosporen von Aspergillus orycae. Der Test eignet sich wegen seiner einfachen Durchführbarkeit besonders für Routine-Untersuchungen.

Literatur

[1] v. Schelhorn, M., Zbl. Bakteriol., Parasitenkunde Infektionskrankh., Abt. II, **107**, 350 (1952–1954).
[2] Mossel, D. A. A., Food Manufact. **31**, 190 (1956).
[3] Coretti, K., Jahresbericht Bundesforsch.anst. Fleischwirtschaft Kulmbach **1955**, 29.
[4] Mossel, D. A. A., und de Bruin, A. S., Analyst **78**, 37–42 (1953); ref. Z. Lebensmittel-Unters. u. -Forsch. **99**, 163 (1954).
[5] Mossel, D. A. A., Mandersloot, J. G., und Gentis, A. S. S., Netherl. Milk Dairy J. **9**, 63 (1955); ref. Z. Lebensmittel-Unters. u. -Forsch. **107**, 80 (1958).
[6] Koch, J., und Schiffner, G., Die Fruchtsaftindustrie **4**, 3 (1959).
[7] Mossel, D. A. A., Zwart, H., und de Bruin, A. S., Analyst **85**, 410 (1960).
[8] Bernarts, M. J., Z. Lebensmittel-Unters. u. -Forsch. **104**, 405 (1956).
[9] Sandi, E., und Szantha, J., Elelmiszervizsgalati Közlemenyek **6**, 141 (1960).
[10] Del Campo, A., Ind. ital. Conserve **32**, 288 (1957).
[11] Baum, F. und Lamm, H., Ernährungsforschung **4**, 84 (1959).
[12] Raible, K., Z. Lebensmittel-Unters. u. -Forsch. **107**, 501 (1958).
[13] Rehm, H. J., Z. Lebensmittel-Unters. u. -Forsch. **110**, 375 (1959).
[14] Sommer, L., und Halbsguth, W., Forschungsberichte des Wirtschafts- und Verkehrsministeriums Nordrhein-Westfalen, Nr. 411, 1957.
[15] Haehn, M., Biochemie der Gärungen. Verlag Walter de Gruyter & Co., Berlin 1952, S. 434.
[16] v. Schelhorn, M., Z. Lebensmittel-Unters. u. -Forsch. **92**, 256 (1951).
[17] Kahlfeld, F., Nährbodentechnik. Verlag Georg Thieme, Leipzig 1948, S. 59.

Table 3.—Effect of repeated matings on the fecundity of the face fly as measured by the rate of oviposition.

Fly age (days)	No. eggs laid					
	♀♀ only[a]			♀♀ and ♂♂[b]		
	Cage 1	Cage 2	Cage 3	Cage 1	Cage 2	Cage 3
6	48	21	37	1	20	0
7	31	0	27	52	4	61
8	71	178	132	116	120	85
9	77	36	92	75	70	165
10	41	104	41	93	30	45
11	154	115	150	160	143	150
12	41	64	23	56	23	39
13	22	0	13	22	0	8
14	127	217	132	125	195	121
15	14	0	0	1	0	3
Total	626	735	647	701	605	677
Eggs/♀/day		10.0			9.9	

[a] Each cage contained 20 ♀.
[b] Each cage contained 20 ♀ and 20 ♂.

rate of oviposition between these flies and those which had mated once and had no further contact with the males. Presumably 1 mating is sufficient to stimulate the flies to sustain normal egg production, and repeated matings do not provide an additional stimulus for oviposition. After mating occurs, the presence of males is not necessary to maintain oviposition by the face fly. Killough and McKinstry (1965) reported that virgin female stable flies, when caged with males from emergence until death, laid more fertile eggs than did the females caged with males for a short period. Thus, there is a species difference in the effects of repeated matings on the rate of oviposition.

REFERENCES CITED

Ascher, K. R. S., and Z. H. Levinson. 1956. The influence of protein addition to the larval diet on oviposition of the house fly, *Musca domestica vicina*. Riv. Parassitol. Rome. 17 (4): 217–22.

Callahan, R. F. 1962. Effects of parental age on the life cycle of the house fly, *Musca domestica* L., (Diptera: Muscidae). J. N. Y. Entomol. Soc. 70: 150–8.

Glasser, R. W. 1923. The effect of food on longevity and reproduction in flies. J. Exp. Zool. 38: 383–412.

Greenberg, G. 1959. House fly nutrition. J. Cell Comp. Physiol. 53 (2): 169–77.

Killough, R. A., and E. S. McClellan. 1965. Face fly oviposition studies. J. Econ. Entomol. 58 (4): 716–9.

Killough, R. A., and D. M. McKinstry. 1965. Mating and oviposition studies on the stable fly. Ibid. 58 (3): 489–91.

Kobbayashi, H. 1934. The influence of foods on the fecundity of *Musca domestica*. Keio J. Med. 5: 36–67.

Larsen, J. R., R. E. Pfadt, and L J. Peterson. 1966. Olfactory and oviposition responses of the house fly to domestic manures, with notes on the autogenous strain. J. Econ. Entomol. 59 (3): 610–5.

Robbins, W. E., and T. J. Shortino. 1962. Effect of cholesterol in larval diet on ovarian development in the adult house fly. Nature (London) 194: 502–3.

Spiller, D. 1964. Nutrition and diet of muscoid flies. Bull. World Health Organ. 31: 551–4.

Turner, E. C., and J. A. Hair. 1967. Effect of diet on longevity and fecundity of laboratory-reared face flies. J. Econ. Entomol. 60 (3): 857–60.

Valder, S. N., and T. L. Hopkins. 1968. Effect of nutrition on egg development and survival in the face fly, *Musca autumnalis*. Ann. Entomol. Soc. Amer. 61 (4): 827–34.

Wang, C. M. 1964. Laboratory observations on the life history and habits of the face fly, *Musca autumnalis* (Diptera: Muscidae). Ibid. 57 (5): 563–9.

Effects of Streptomycin and Potassium Sorbate Levels in Relation to Nutrient Levels on the Larvae of *Agria affinis*[1,2]

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ABSTRACT

Feeding 4 levels of nutrients with 4 levels of streptomycin and 4 levels of potassium sorbate, respectively, to larvae of *Agria affinis* auct. nec Fallén showed that effects of both antimicrobial agents on growth, development, and survival depend upon the concentration of the agents and upon the nutrient level. Without either streptomycin or potassium sorbate the rate of growth, development, and survival were the same on all levels of nutrients. With increasing levels of either streptomycin or potassium sorbate in the diet, the rates of growth, development, and survival decreased. However, these effects decreased with increasing levels of nutrients.

This work shows the effects on the larvae of *Agria affinis* auct. nec Fallén of streptomycin and potassium sorbate levels in relation to dietary nutrient levels.

It is customary to use antibiotics and mold inhibitors to control microbial contamination of synthetic diets for insects. A review of literature shows that more than 30 antimicrobial agents, separately or combined, have been used in about 100 synthetic diets to control microbial contamination. However, the desirability of this practice has been questioned because of possible direct toxic or repellant effects on the insect itself, or of toxic effects on the beneficial microorganisms that are harbored in the gut and which may aid digestion or provide supplementary sources of nutrients.

For example, Beck et al. (1949) and Fytizas and Tzanakakis (1966) reported no toxic effects of methyl *p*-hydroxy benzoate, butyl *p*-hydroxy benzoate, or streptomycin in the artificial diets of the European corn borer, *Ostrinia nubilalis* (Hübner), and adults of the olive fruit fly, *Dacus oleae* (Gmelin), respec-

[1] Diptera: Sarcophagidae.
[2] Received for publication June 16, 1969

Table 1.—Composition of 4 diets for rearing *A. affinis* larvae axenically[a]; gm/100 ml of diet.

% nutrient in diet	Amino acid mix	Salt mix	RNA	Lipid mix	Tween 80	Glucose	B-vitamin mix	KOH	Agar
100	4.0	0.132	0.1504	1.00	0.50	1.00	0.0220	0.314	0.75
75	3.0	.099	.1128	0.75	.37	0.75	.0165	.235	.75
50	2.0	.066	.0752	.50	.25	.50	.0110	.157	.75
25	1.0	.033	.0376	.25	.12	.25	.0055	.078	.75

[a] House's (1967) diet A.

tively. On the other hand, Kishaba et al. (1968) and Vail et al. (1968) for the cabbage looper, *Trichoplusia ni* (Hübner), and Clark et al. (1961) and Ouye (1962) for the pink bollworm, *Pectinophora gossypiella* (Saunders), found that most antimicrobial agents were toxic or detrimental. Similar results were reported by Prokopy (1967) for the apple maggot, *Rhagoletis pomonella* (Walsh), with sodium benzoate or methyl *p*-hydroxy benzoate. Our preliminary experiment, using *A. affinis* larvae, gave conflicting results when certain antibiotics and mold inhibitors were tested on diets of different nutrient composition. Therefore, work was initiated at Belleville to determine the levels of antibiotics and mold inhibitors that have no apparent effects on the insect, i.e. "safe levels," for use in insect dietetics. This report deals with a portion of these investigations.

MATERIALS AND METHODS.—Detailed accounts of the composition and preparation of the diets and the equipment and techniques for handling and rearing *A. affinis* axenically for nutritional assays are described elsewhere (House 1954, 1967; House and Barlow 1956, 1957, 1960, 1965).

The dietary components were those used previously, i.e. diet A (House 1967). In the present work, the nutrients used were qualitatively the same in all diets. Quantitatively, the amount of each nutrient was varied at the same rate, as in Table 1, so that the diets contained 75, 50, and 25% of the levels of each nutrient contained in the 1st diet; these are referred to as the 100, 75, 50, and 25% diets, respectively. Each diet was prepared in 2 parts. The 1st part consisted of dry ingredients—amino acids, RNA, salt mixture, and agar—weighed separately into a flask and dissolved in the lipid mixture, 2N potassium hydroxide and water. The agar level was kept constant and the amount of 2N KOH was varied independently to adjust the *p*H of diets to about 6.5. Then a predetermined amount of water was added to this part of the diet (i.e. so that the total water of this part combined with that of the 2nd part—the aqueous of glucose and vitamins—and that of the antimicrobial agent finally added (see below) resulted in a total volume of 100 ml). This part of the diet w autoclaved. The 2nd part of the diet consisted glucose/vitamin mixture, which was sterilized by fi tering. The 2 parts of the diets were then combine under aseptic conditions.

Solutions of the antimicrobial agents used we made in concentrations that delivered to the di given amounts of the agent per ml (see below) These solutions were sterilized by filtering. Upo combining the 2 parts of the diets, predetermine volumes of the antimicrobial agent were added; th completed each diet and brought its volume to 10 ml. The streptomycin (British Drug Houses (Ca ada) Limited) used was a streptomycin sulpha solution, 0.5 gm streptomycin base/ml. The pota sium sorbate used assayed 99.0% (Matheson Colema & Bell, Division of Matheson Co. Inc., Norwoo (Cincinnati), Ohio).

Feeding tests were done with larvae dissected ase tically from gravid females and randomly distribute to the different diets. One larva was put in each te tube containing 1 ml of diet. The larvae were e amined daily in the test tubes without breakin asepsis until the 10th day, inclusive, when the mo tality and numbers of larvae in the 2nd and 3rd i star were recorded. The diets were tested 3 times wi 25 larvae/diet per test at 23±1°C.

Rates of growth and development on various die were evaluated by analysis of variance (Snedeco 1946) and multiple range test (Duncan 1955). Th rates of growth, expressed as percentage of axeni larvae that had reached the 2nd instar by the 4t day for streptomycin and about 5th day for potassiu sorbate, were first transformed to $\sin-1\sqrt{\%}$ and the subjected to an analysis of variance. Differences we considered significant when the probability of F wa less than 0.05. Mortality data based on eye-fitte method are expressed on a log-dosage probabilit curve.

Table 2.—Rate of growth and development of *A. affinis* larvae of 4 nutrient levels, expressed as the percent of larvae that attained 2nd instar in 4 days; average of 3 tests of 25 larvae per diet per test.

Percent nutrient in diet	Percent
100	98.7
75	97.3
50	96.0
25	88.0

Table 3.—Effects of 4 levels of nurtrients and 4 level of streptomycin on the rate of growth and developme of *A. affinis* larvae, expressed as the percent of larva that attained 2nd instar in 4 days; average of 3 tes of 25 larvae per diet per test.

% nutrient in diet	Streptomycin mg/100 ml of diet				
	800	1600	2400	3200	Mea
100	93.3	76.0	54.7	37.3	65.3
75	88.0	44.6	30.7	13.6	44.2
50	66.7	22.7	6.7	2.7	24.7
25	0.0	0.0	0.0	0.0	0.0
Mean	62.0	35.8	23.0	13.4	

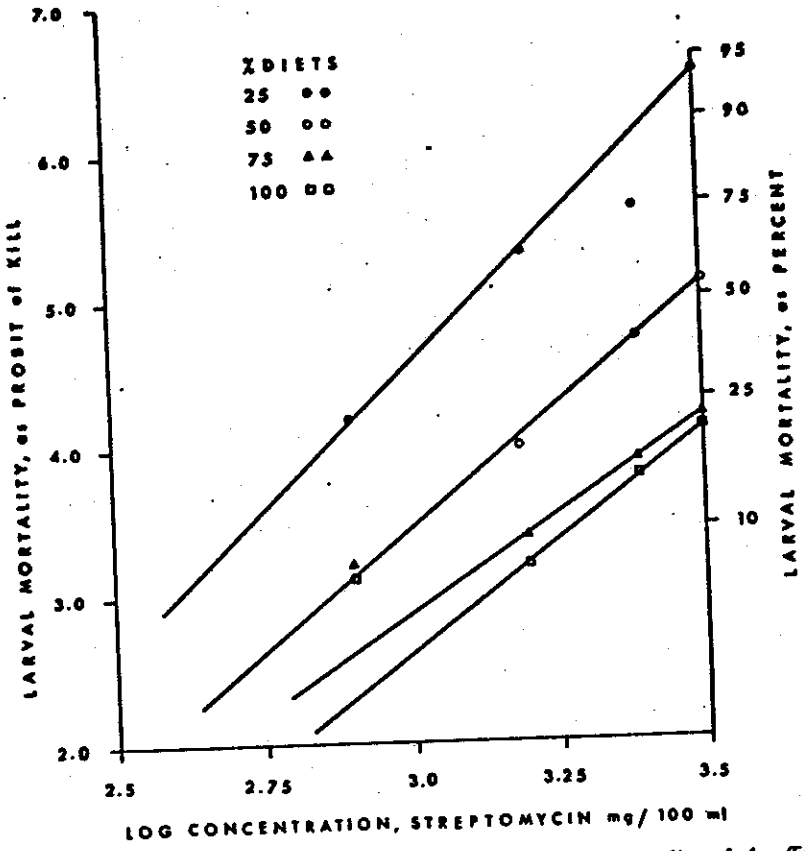

FIG. 1.—Effects of 4 levels of nutrients and 4 levels of streptomycin on the mortality of *A. affinis* larvae: cumulative mortality within 10 days; average of 3 tests of 25 larvae/diet per test.

RESULTS.—*Rate of Growth on 4 Levels of Dietary Nutrients.*—Four diets (100, 75, 50, and 25%) were prepared as just described. No streptomycin or potassium sorbate was added to these diets. The rates of growth of larvae and mortality were determined.

Table 2 shows the percent of 2nd-instar larvae on the 4th day. Statistical analysis on the rate of growth and development of the larvae showed no significant differences between nutrient levels.

The ET_{50} (time, measured to the nearest day, required for 50% larvae to reach a particular instar) of 2nd-instar larvae on all 4 diets was 2.2 days, and similarly for 3rd instar the ET_{50} was 4.5 days. The natural mortality on the 4 diets during 10 days was negligible (0–4%).

Effects of 4 Levels of Dietary Nutrients and 4 Levels of Streptomycin.—An experiment was done with 4 levels of dietary nutrients i.e. 100, 75, 50, and 25% in all combinations with 4 levels of streptomycin used at the rate of 800, 1600, 2400, and 3200 mg/100 ml of diet, respectively.

Results of analysis (Table 3) show that differences in the rate of growth and development of the 2nd-instar larvae reared on different nutrient and streptomycin levels were statistically significant; moreover, there was a significant interaction between them. For example, rate of growth and development decreased with decreasing levels of nutrients and with increasing levels of streptomycin. Thus, the effects of streptomycin were not uniform over all the levels of nutrients. On all levels of streptomycin no larvae developed to the 2nd instar on the lowest nutrient level. Differences in the rate of growth and development were statistically significant between all levels of streptomycin except between 1600 and 2400 and between 2400 and 3200 mg/100 ml of diet.

Fig. 1 shows larval mortality at the 4 levels of streptomycin in conjunction with the 4 nutrient levels. Mortality varied directly with the concentrations of streptomycin and indirectly with the nutrient concentration.

The ET_{50} of the 2nd-instar larvae was 5–6 days, irrespective of dietary nutrient or streptomycin levels.

Table 4.—Effects of 4 levels of nutrients and 4 levels of Potassium sorbate on the rate of growth and development of *A. affinis* larvae expressed as the percent of larvae that attained 2nd instar in about 5 days: average of 3 tests of 25 larvae per diet per test.

% nutrient in diet	Potassium sorbate mg/100 ml diet				
	800	900	1000	1100	Mean
100	97.3	97.3	97.3	86.7	94.6
75	90.4	89.3	80.0	76.0	83.9
50	53.3	36.0	21.3	0.0	27.6
25	0.0*	0.0	0.0	0.0	0.0
Mean	60.2	55.6	49.6	40.7	

Effects of 4 Levels of Dietary Nutrients and 4 Levels of Potassium Sorbate.—An experiment was done with 4 levels of dietary nutrients, i.e. 100, 75, 50, and 25% in all combinations with 4 levels of potassium sorbate used at the rate of 800, 900, 1000, and 1100 mg/100 ml of diet, respectively.

Results shown in Table 4 indicate that differences in the rate of growth and development of the 2nd-instar larvae varied on different nutrient and potassium sorbate levels, and were statistically significant; moreover, there was a significant interaction between them. For example, rate of growth and developmen decreased with decreasing levels of nutrients and with increasing levels of potassium sorbate. Thus, the effects of potassium sorbate were not uniform over all the levels of nutrients. On all levels of potassium sorbate no larvae developed on the lowest nutrient level. Differences in the rate of growth and development were statistically significant between all levels of potassium sorbate except between 800 and 900, between 900 and 1000, and between 1000 and 1100 mg/100 ml of diet.

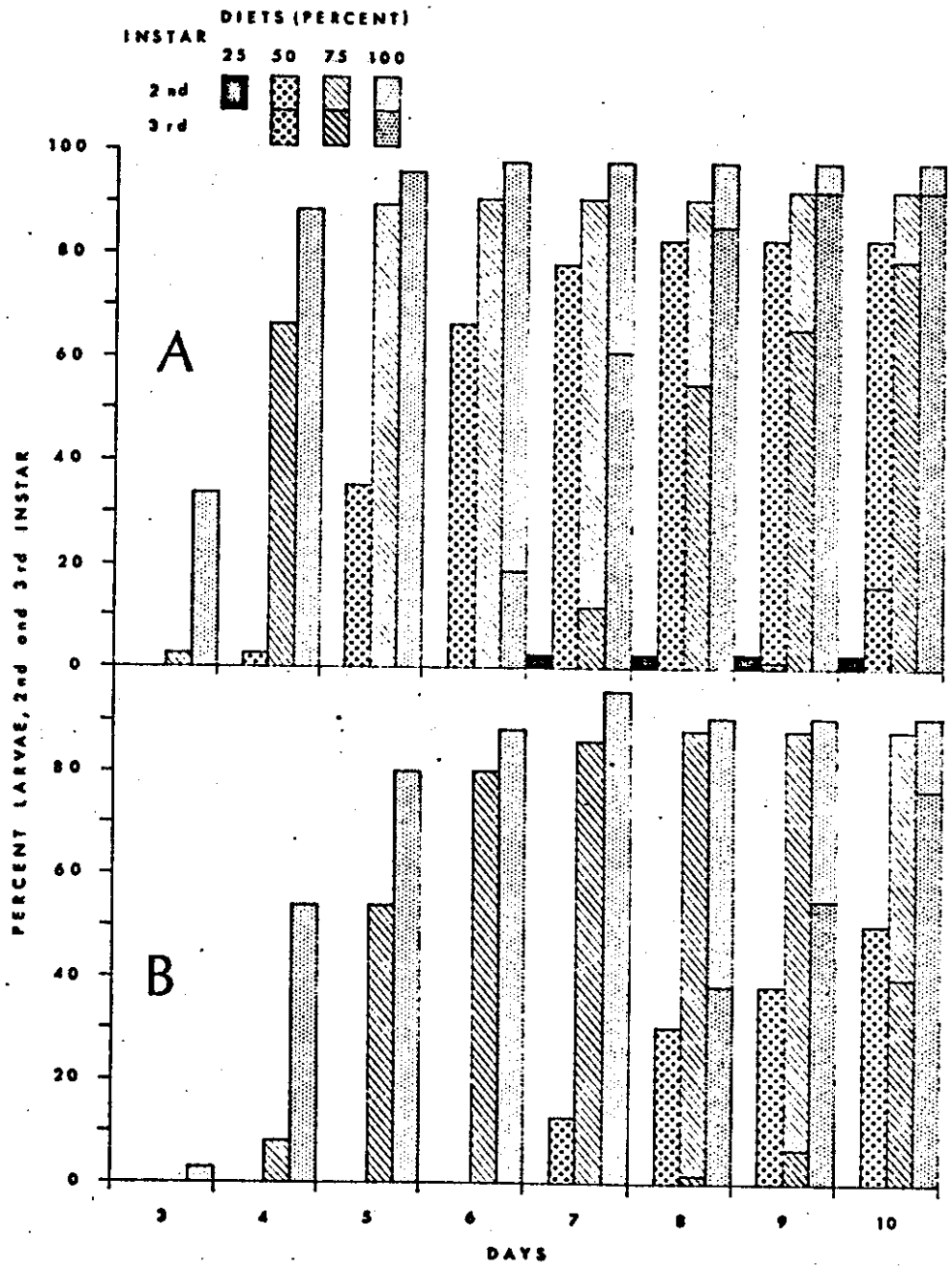

FIG. 2.—Rate of growth and development of *A. affinis* larvae on 4 levels of nutrients with 800 A and 1100 B mg potassium sorbate/100 ml of diet; average of 5 tests of 25 larvae/diet per test.

The effects of various levels of potassium sorbate on larval mortality varied between nutrient levels. The maximum was 33.3% on 1000 mg/100 ml of 50% diet; on 100, 75, and 25% diets it varied between 1.3 to 13.0%, irrespective of potassium sorbate levels.

Fig. 2 A shows the effects of potassium sorbate on the rate of growth and development of larvae. With 800 mg of potassium sorbate, on 2nd-instar larvae some differences in the rate of growth and development between diets occurred during the 1st week, but later the differences narrowed and within 10 days growth and development were similar on all diets except on the 25% diet, which did not catch up. Similar results, Fig. 2 B, were obtained with 1100 mg of potassum sorbate, but effects were more severe, and only 51% larvae reached the 2nd instar on the 50% diet and none on the 25% diet.

Moreover, Fig. 2 A, B, shows the effects of potassium sorbate on the rate of growth and development measured to 3rd-instar larvae. Here too, the rate of growth and development varied directly with increasing nutrient levels and inversely with increasing potassium sorbate levels.

The ET_{50} of 2nd-instar larvae was 5 to 6 days, irrespective of dietary nutrient or potassium sorbate levels.

DISCUSSION.—This work showed that the presence of streptomycin and potassium sorbate in the diets had adverse effects on the rate of growth, development, and survival of *A. affinis* larvae. Such effects increased with increasing the levels of streptomycin or potassium sorbate, and with decreasing the levels of nutrients. Some other work has reported that antimicrobial agents are detrimental in high concentrations. For example, high concentrations of butyl *p*-hydroxy benzoate, methyl *p*-hydroxy benzoate, and sorbic acid increased the time required for larval development in the cabbage looper and the pink bollworm (Kishaba et al. 1968, Ouye 1962). Moore et al. (1967) reported that a mixture of methyl *p*-hydroxy benzoate and potassium sorbate in the larval diet of boll weevil, *Anthonomus grandis* Boheman, had no adverse effects, but when added to larval and adult diet, egg production and the yield of adults decreased; however, decreasing the amounts of these mold inhibitors by 50%, the length of the larval development decreased and the yield of adults increased. Similarly, Toba et al. (1969) could improve considerably the rearing of pepper weevil, *Anthonomus eugenii* Cano, by decreasing the amount of mold inhibitor in the diet.

Moreover, the effects of antimicrobial agents in insect dietetics depend on the type and level of nutrients. Clark et al. (1961) used 2 sources of protein, peanut flour and cotton seed meal, in the diet of pink bollworm and reported that the effects of mold inhibitors depend on source of protein used. Fytizas and Tzanakakis (1966) found no effect of streptomycin on adults of the olive fruit fly reared on an artificial diet. However, feeding streptomycin to the parental females inhibited growth of their progeny in olive fruits that were fresh or stored up to 3 weeks, but not on fruits stored longer probably because free amino acids accumulated in the mesocarp during prolonged storage. This result suggests that change in nutrient content of the food might have offset, or changed the potency of streptomycin.

We conclude that addition of streptomycin or potassium sorbate to the diets of *A. affinis* larvae is detrimental and that the various effects on the larvae are modified by the nutrient level of the food medium. It is important then to consider nutrient levels when antimicrobial agents are used. For a given diet may accommodate only a given concentration of the antimicrobial agent before adversely affecting the insect concerned. In other words: apparently the safe level of an antimicrobial agent depends on the nutrient level, the concentration and kind of antimicrobial agent, and perhaps on the species or kind of insects.

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REFERENCES CITED

Beck, S. D., J. H. Lilly, and J. F. Stauffer. 1949. Nutrition of European corn borer, *Pyrausta nubilalis* (Hbn.). I. Development of a satisfactory purified diet for larval growth. Ann. Entomol. Soc. Amer. 42: 483–96.

Clark, E. W., C. A. Richmond, and J. M. McGough. 1961. Artificial media and rearing techniques for the pink bollworm. J. Econ. Entomol. 54: 4–9.

Duncan, D. B. 1955. Multiple range and multiple F tests. Biometrics 11: 1–41.

Fytizas, E., and M. E. Tzanakakis. 1966. Some effects of Streptomycin when added to the adult food, on the adults of *Dacus oleae* (Diptera: Tephritidae) and their progeny. Ann. Entomol. Soc. Amer. 59: 269–73.

House, H. L. 1954. Nutritional studies with *Pseudosarcophaga affinis* (Fall.), a dipterous parasite of the spruce budworm, *Choristoneura fumiferana* (Clem). I. A chemically defined medium and asepetic culture technique. Can. J. Zool. 32: 331–41.

1967. The role of nutritional factors in food selection and preference as related to larval nutrition of an insect, *Pseudosarcophaga affinis* (Diptera, Sarcophagidae), on synthetic diets. Can. Entomol. 99: 1310–21.

House, H. L., and J. S. Barlow. 1956. Nutritional studies with *Pseudosarcophaga affinis* (Fall.), a dipterous parasite of the spruce budworm, *Choristoneura fumiferana* (Clem.). V. Effects of various concentrations of the amino acid mixture, dextrose, potassium ion, the salt mixture, and lard on growth and development; and a substitute for lard. Can. J. Zool. 34: 182–9.

1957. New equipment for rearing small numbers of *Pseudosarcophaga affinis* (Fall.) (Diptera: Sarcophagidae) for experimental purposes. Can. Entomol. 89: 145–50.

1960. Effects of oleic and other fatty acids on the growth rate of *Agria affinis* (Fall.) (Diptera: Sarcophagidae). J. Nutr. 72: 409–14.

1965. The effects of a new salt mixture developed for *Agria affinis* (Fallén) (Diptera: Sarcophagidae) on the growth rate, body weight and protein content of the larvae. J. Insect Physiol. 11: 915–8.

Kishaba, A. N., T. J. Henneberry, R. Pangaldan, and P. H. Tsao. 1968. Effects of mould inhibitors in larval diet on the biology of Cabbage looper. J. Econ. Entomol. 61: 1189–94.

Moore, R. F. Jr., F. F. Whisnant, and H. M. Taft. 1967. A laboratory diet containing egg albumin for larval and adult boll weevils. Ibid. 60: 237–41.

Ouye, M. T. 1962. Effect of antimicrobial agents on microorganisms and pink bollworm development. Ibid. 55: 854–7.

Prokopy, R. J. 1967. Artificial diets for apple maggot larvae. Ibid. 60: 1161–2.

Snedecor, G. W. 1946. Statistical Methods. 4th ed. Iowa State College Press, Ames. 485 p.

Toba, H. H., A. N. Kishaba, R. Pangaldan, and S. Riggs. 1969. Laboratory rearing of pepper weevils on artificial diets. J. Econ. Entomol. 62: 257–8.

Vail, P. V., T. J. Henneberry, A. N. Kishaba, and A. Y. Arakawa. 1968. Sodium hypochloride and formalin as antiviral agents against nuclear polyhedrosis virus in the larvae of cabbage looper. J. Invertbr. Pathol. 10: 84–93.

Mating Competitiveness of Mediterranean Fruit Flies[1] Marked with Fluorescent Powders[2,3]

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ABSTRACT

In tests made in Hawaii in outdoor screen cages, the mating competitiveness of laboratory-reared *Ceratitis capitata* (Wiedemann) was not altered by external marking with orange, yellow, or green Day-glo® fluorescent powders. Also, adults of both sexes marked with red powder had an increased mating response. Most mating occurred in the downwind corners of the cages and on the wooden supports rather than on the screen.

Various physical, chemical, and radioactive techniques have been used to mark insects for use in release-recapture studies. For example, an oil-soluble dye was the marker for the pupae of the melon fly, *Dacus cucurbitae* Coquillet, that were used for calculating the overflooding ratios of adults necessary to eradicate that species from Rota, Marianas Islands (Steiner et al. 1965). However, this dye was difficult to see on older flies, and it was necessary to crush their heads to be sure they were the adults from the marked pupae (Steiner 1965). Subsequently, Medley and Ahrens (1968) and Vail et al. (1966) used fluorescent powders as insect markers and found that they could be quickly identified with an UV lamp. The following tests were made at the Hawaiian Fruit Flies Investigations Laboratory at Honolulu, in 1968, to determine the effects of 4 Day-glo® (Switzer Brothers Inc., Cleveland, Ohio 44103) fluorescent powders on the mating competitiveness of male and female Mediterranean fruit flies, *Ceratitis capitata* (Wiedemann). These tests were part of a series being made to evaluate handling techniques and their effects on fruit flies intended for use in sterile-release eradication programs.

METHODS AND MATERIALS.—The fluorescent powders selected for testing were Blaze Orange, Rocket Red, Signal Green, and Arc Yellow. Two large (9×9×8-ft) outdoor screened cages were available, so that 2 powders could be tested simultaneously. A total of 12,000 puparia was used to provide adults for 3 tests/week of the 2 powders being tested. Marking was accomplished by distributing 0.1 g of each powder over each of 2 lots of 3000 puparia 2 days before eclosion; the remaining 6000 (controls) were left unmarked.

The lots of 3000 puparia (2 lots marked and 2 lots unmarked) were placed in separate no. 12 Kraft paper bags containing X-shaped cardboard inserts (to increase the resting area and prevent collapse of the bags), and 3 sugar cubes each for food. At first, the bags were placed on shelves in a room which had lighting on a normal daily cycle. Dissections of the spermathecae indicated that a small amount of mating (less than 10%) took place in the bags within 48 hr of emergence. To prevent this mating, inhibit excitation of the flies, and facilitate handling, the bags were thereafter placed in a closed corrugated cardboard box and held in darkness; at ca. 28°C and 50% RH. Two days after adult emergence, the bags were opened and the adults were allowed to fly from a bag into a 1-ft cubical screen cage which was then held at 7°C for ca. 5 min (or until the flies were immobilized). Four lots of 100 adults of each sex and each treatment (a total of 16 lots) and an equal number of unmarked (control) flies were placed in separate 1-qt screened-top cardboard containers and provided with sugar, water, and Type M hydrolized protein (Nutritional Biochemicals Corp., 26201 Miles Rd., Cleveland, Ohio 44128). They were then held at ca. 27°C and 65–75% RH for 6–8 days to assure sexual maturity and full mating response.

The mating tests were made in the two 9×9×8-ft screened outdoor cages, a separate cage for 100 each of each color of marked males and females, together with an equal number of unmarked males and females. However, the males were released before the females, and the holding containers were removed from the vicinity of the large cages, because the females would congregate on the containers of males or even on the containers that had recently held males (they did not respond to containers that had been empty of males for 24 hr).

The mating tests were begun at about 8:30 AM. As soon as mating commenced, pairs in copula were collected individually until either 100 had been taken or no matings had occurred for 30 min. (The 100 pairs were often collected in less than 1 hr, but no test was extended beyond 1 PM.) After each test, all remaining flies were removed from the cages. The marking of each member of each mated pair was determined under UV light and recorded in 1 of 4 possible mating categories: unmarked male × unmarked female; unmarked male × marked female; marked male × unmarked female; and marked male × marked female. If less than 100 mated pairs were collected the frequencies in each mating category were transformed to percentages. All numbers could then be transformed by using the arcsin method before

[1] Diptera: Tephritidae.

[2] Received for publication June 27, 1969.

[3] Mention of a proprietary product does not necessarily imply its endorsement by the USDA.

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INHIBITION OF ALCOHOLIC FERMENTATION BY SORBIC ACID[1]

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Sorbic acid (2,4-hexadienoic acid) is the most effective of a number of α,β-unsaturated monocarboxylic fatty acids reported to inhibit the fungi (Gooding, 1945). It has been studied extensively with respect to inhibitory activity on various groups of microorganisms; and its use in controlling fermentative yeasts in cucumber fermentations has been the subject of considerable investigation in this laboratory (Costilow et al., 1955, 1957). Although this acid is a very effective inhibitor of yeast fermentation, the actual enzyme(s) inhibited have not been clearly elucidated. The present study was undertaken to determine the site(s) of sorbic acid inhibition in the anaerobic metabolism of *Saccharomyces cerevisiae*.

Melnick, Luckmann, and Gooding (1954) suggested that sorbic acid inhibited dehydrogenase systems in molds, and York and Vaughn (1955) reported it to be active in suppression of fumarase action. Both of these reports were concerned with aerobic metabolism and shed no light on the activity of this compound in anaerobic systems. Recently, Whitaker (1959) demonstrated that crystalline alcohol dehydrogenase was inhibited after incubation with sorbic acid. He suggested that the action on microorganisms is similar to that of maleic acid; i.e., the forming of stable complexes with sulfhydryl containing enzymes. Data collected in these studies are not in agreement with this hypothesis.

MATERIALS AND METHODS

The yeast used in these experiments was baker's yeast purchased locally, air dried, and maintained in the freezer. The cell-free extracts were prepared by blending a mixture of 15 g of dried yeast, 45 g of Superbrite glass beads[2] (average size, 0.2 mm), and 50 ml of water in a Servall omnimixer. The mixture was cooled in an ice bath before blending and held in the bath during a 5-min blending period. The beads, intact cells, and cell debris were removed by centrifugation in the cold. This method resulted in a high percentage of cell disruption and yielded an extract with good fermentative activity. However, it was necessary to add appropriate cofactors to maintain constant fermentation rates with various substrates.

Warburg techniques were used to measure CO_2 production by the yeast extracts from glucose (0.01 M), fructose 1,6-diphosphate (0.13 M), and 3-phosphoglyceric acid (0.01 M). These were all run in 0.033 M phosphate buffer at pH 6.0. Glucose fermentation was also tested at pH 5.0 with 0.033 M phthalate buffer. A mixture containing 5 μmoles adenosine triphosphate (ATP), 2 μmoles adenosine diphosphate (ADP), 10 μmoles, $MgCl_2$, and 2 μmoles diphosphopyridine nucleotide (DPN) was added to each Warburg cup along with 0.5 ml of the cell-free extract to study glucose fermentation. With fructose 1,6-diphosphate as substrate, the ATP was omitted from the reaction mixtures; and with 3-phosphoglyceric acid, both the ATP and DPN were omitted and 2 μmoles of reduced diphosphopyridine nucleotide (DPNH) were added. The total volume of liquid in each cup was adjusted to 3 ml, and the reactions run in a nitrogen atmosphere.

Activity of yeast extract with phosphoenolpyruvate as substrate was determined by measuring the rate of DPNH oxidation spectrophotometrically at 340 mμ. The crude yeast extract was dialyzed overnight against distilled water in the cold before running the assay. Reaction mixtures contained 0.5 ml of dialyzed yeast extract, phosphoenolpyruvate (8×10^{-4} M), ADP (3.3×10^{-4} M), $MgSO_4$ (8×10^{-3} M), and DPNH (1.5×10^{-4} M) in 0.067 M phosphate buffer (pH 7.4). The volume of the liquid in the cuvette was adjusted to 3 ml with distilled water.

Possible phosphoglyceromutase inhibition was tested indirectly by blocking enolase with fluoride in dialyzed yeast extract and measuring DPNH

[1] Journal article no. 2497.

[2] Manufactured by Minnesota Mining and Manufacturing Company, St. Paul.

oxidation with 2-phosphoglyceric acid as substrate. The methods used were as described in the preceding paragraph except that the reaction mixture contained 0.05 ml of dialyzed yeast extract, 2-phosphoglyceric acid (4×10^{-4} M), ATP (2×10^{-4} M), $MgSO_4$ (8×10^{-3} M), DPNH (3.6×10^{-4} M), and NaF (0.16 M) in 0.05 M imidazole buffer (pH 7.4). When the ATP was replaced with ADP in the mixture, no oxidation of DPNH occurred indicating that the fluoride blocked the enolase activity completely.

Carboxylase activity was measured by the method of Green, Herbert, and Subrahmanyan (1941) and alcohol dehydrogenase by that of Racker (1950) except that in both instances the crude yeast extract was used without purification. The effect of sorbic acid was also tested on a commercial preparation of alcohol dehydrogenase by the same method.

Possible inhibition of the aldolase or 3-phosphoglyceraldehyde dehydrogenase of the yeast extract was tested by the method of Warburg and Christian (1943) for determining aldolase activity, except that no purification procedures were used and no 3-phosphoglyceraldehyde dehydrogenase was added to the reaction mixture.

Enolase was determined by the method of Warburg and Christian (1941) as modified by Wold and Ballou (1957). With 2-phosphoglyceric acid as substrate, the appearance of phosphoenolpyruvate is followed by measuring the increase in optical density at 240 mμ. A Beckman model DU spectrophotometer was used for all spectrophotometric work.

Refined sorbic acid, obtained from the Carbide and Carbon Chemicals Company, was recrystallized three times from distilled water before use. Fresh stock solutions (1.5×10^{-2} M) were maintained in the refrigerator during the experiments. These were adjusted to the pH level desired for each experiment and diluted to give a final concentration of 5×10^{-3} M in all inhibition studies except for those with enolase. This was much too concentrated for the enolase work, and a number of lower concentrations were used.

The cofactors and glycolytic-intermediate compounds used were of commercial origin, and solutions were either prepared fresh for each experiment or maintained in the frozen state.

RESULTS AND DISCUSSION

Initial experiments demonstrated that the fermentation of glucose by resting cells of baker's yeast was greatly inhibited by sorbic acid. As expected from previous work (Bell, Etchells, and Borg, 1959), the extent of the inhibition was very dependent on the pH. At pH 6.0, the fermentation was inhibited 25 per cent, whereas 46 per cent inhibition was observed at pH 5.0. In an attempt to ascertain the reason for this effect of pH, cell-free extracts of yeast were prepared and used for inhibition studies. As shown in figure 1, glucose fermentation was inhibited 26 per cent at pH 6.0 and 29 per cent at pH 5.0.

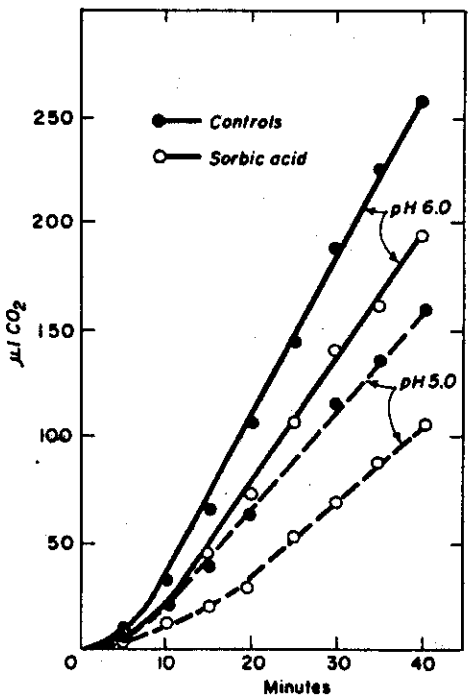

Figure 1. Effect of sorbic acid (5×10^{-3} M) on glucose fermentation by cell-free extracts of yeast at two pH levels as measured by CO_2 evolution. The main compartment of each Warburg cup contained 5 μmoles ATP, 2 μmoles ADP, 2 μmoles DPN, 10 μmoles $MgCl_2$, 0.5 ml of 0.2 M buffer (phosphate buffer used for pH 6.0 and phthalate buffer for pH 5.0), and 0.5 ml extract. Glucose, 0.5 ml of 0.06 M, was tipped in from a side arm after thermal equilibrium had been attained at 30 C. One milliliter of 0.015 M sorbic acid adjusted to the reaction pH was added to main compartments of cups for inhibition tests. The total volume was 3.0 ml. The flasks were gassed with N_2.

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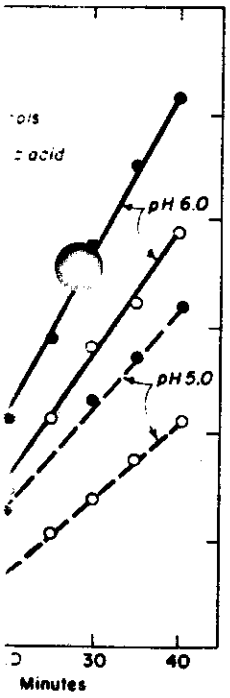

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Thus, there was no significant difference in the per cent inhibition at the two pH levels. These data support the hypothesis of Bell et al. (1959) that the enhanced inhibitory action of this acid at low pH levels is primarily due to the greater permeability of the cell to the undissociated form.

To determine the specific enzyme(s) inhibited by sorbic acid, tests for inhibition of activity of cell-free extracts with a number of glycolytic intermediates as substrates were conducted. The rationale behind these experiments was to limit the number of possible inhibitory sites.

First, a study was conducted of the influence of sorbic acid on the rates of CO_2 production from fructose 1,6-diphosphate and 3-phosphoglyceric acid. Manometric studies demonstrated that CO_2 production was inhibited by 28 per cent with the former and by 26 per cent with the latter substrate. Since the degree of inhibition was the same as with glucose, it was indicated that the site of sorbic acid inhibition was beyond 3-phosphoglyceric acid in the Embden-Meyerhof sequence. This was substantiated by the fact that there was no inhibition of DPN reduction by these crude yeast extracts using fructose 1,6-diphosphate as substrate, indicating that neither aldolase or 3-phosphoglyceraldehyde dehydrogenase were affected greatly. The range of possible sites was further narrowed by testing the effect of sorbic acid on carboxylase and alcohol dehydrogenase activity of cell-free yeast extract. No inhibition of these two enzymes was observed. Neither was there any indication of phosphopyruvic acid transphosphorylase inhibition since sorbic acid did not influence the rate of DPNH oxidation by dialyzed crude yeast extract with phosphoenolpyruvate as substrate. Phosphoglyceromutase is probably not affected by sorbic acid since there was no inhibition of DPNH oxidation by dialyzed yeast extract with 2-phosphoglyceric acid as substrate in the presence of ATP and sufficient fluoride to inhibit enolase completely. Therefore, it was concluded that enolase was the most likely site of sorbic acid inhibition of alcoholic fermentation.

Since there are a number of the α,β-unsaturated fatty acids which are quite inhibitory to yeasts, whereas their saturated counterparts are ineffective at similar concentrations, and, since phosphoenolpyruvate is unsaturated in the α,β position, it appeared that a competition between sorbate and phosphoenolpyruvate might occur. While 2-phosphoglyceric acid is the substrate for enolase in the normal fermentation sequence, the Michaelis constants for the forward and reverse reaction are about equal according to Wold and Ballou (1957); therefore, the affinity of the enzyme for 2-phosphoglyceric acid and phosphoenolpyruvate is about the same, and competition with the product would be expected to cause inhibition.

To test this hypothesis, purified yeast enolase was obtained through the generosity of Dr. Finn Wold, Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, and tested for sorbic acid inhibition. These tests were conducted using a constant enzyme concentration of 6.0×10^{-10} M and various sorbic acid and substrate (2-phosphoglyceric acid) concentrations.

Sorbic acid concentrations of 1.5×10^{-4} and higher resulted in definite inhibition of enolase activity with a substrate concentration of 2×10^{-4} M (figure 2). Concentrations of sorbic acid below 1.5×10^{-4} M resulted in very little or no inhibition of the enzyme at this substrate level; and, when the sorbic acid level was raised much above 2.5×10^{-4} M, no significant enzyme activity could be detected.

Similar experiments conducted at substrate concentrations of 4×10^{-4} and 5×10^{-5} M demonstrated that the substrate concentration had a pronounced effect on the degree of enolase inhibition by a given level of sorbic acid. This is illustrated in figure 3 where the reciprocals of the reaction velocities are plotted versus the reciprocals of substrate concentrations for the various sorbic acid concentrations used (Lineweaver-Burk plot). From this plot it appears that the inhibition is competitive at sorbic acid concentrations up to 2.0×10^{-4} M and is noncompetitive at higher inhibitor levels. Thus, the intercept is constant and the slope increases with increasing inhibitor concentrations up to 2.0×10^{-4} M, whereas both the slope and intercept change at higher concentrations. However, a plot of reciprocal velocity versus inhibitor concentration yields a curve whose slope increases with increasing inhibitor levels, which indicates that the inhibition is partially competitive and partially noncompetitive (Dixon and Webb, 1958). Therefore,

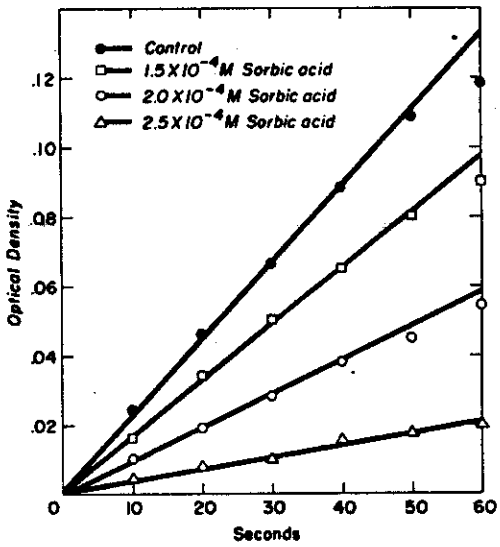

Figure 2. Inhibition of yeast enolase activity by sorbic acid. Activities were measured in a spectrophotometer at room temperature. Each cuvette contained 3 ml of a reaction mixture of the following: 6×10^{-10} M enolase, 2×10^{-4} M substrate (2-phosphoglyceric acid), 8×10^{-3} M $MgCl_2$, and 5×10^{-2} M imidazole buffer at pH 7.4. Appropriate solutions of sorbic acid adjusted to pH 7.4 were added to give the concentrations indicated in the figure. The increase in optical density was recorded at 10-sec intervals.

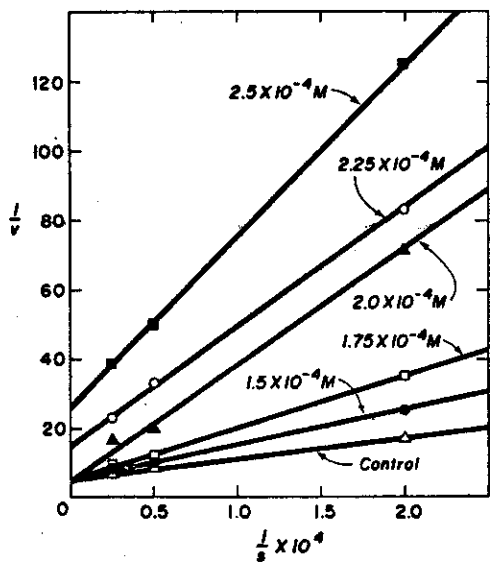

Figure 3. Lineweaver-Burk plot of sorbic acid inhibition of enolase. Inhibition studies were run as described under figure 2 with substrate concentrations of 5×10^{-5} M, 2×10^{-4} M, and 4×10^{-4} M. The reaction velocities (V) were calculated in terms of the change in optical density per min for each sorbic acid-substrate combination, and the reciprocals of the velocities plotted versus the reciprocals of the substrate concentrations for each sorbic acid concentration indicated in the figure.

it is concluded that the sorbic acid inhibition of enolase is a complex type.

Other α,β-unsaturated acids, crotonic (4×10^{-4} M) and cinnamic acid (4×10^{-4} M), were found to inhibit enolase activity. However, they were not as effective as sorbic acid.

In determining enolase activity in the presence of sorbic acid, it was noted that there was an initial decrease in optical density at 240 mμ on the addition of enolase to the reaction mixture. This masked the initial increase in optical density due to enzymatic action and created an apparent lag. The problem was eliminated by adjusting the instrument to zero 10 sec after adding enzyme to the reaction mixture. This decrease in absorbancy probably results from a combination of the sorbate with the enzyme, since the sorbate absorbs light strongly at this wavelength. Further studies showed that this drop was independent of the substrate, but was influenced by the concentrations of sorbic acid, enolase, and Mg^{++}. Up to a point, an increased level of any one of these with a given concentration of the other two, would result in a greater optical density drop. Similar losses in optical density were observed on mixing other α,β-unsaturated acids (crotonic and cinnamic acids) with enolase. Also this phenomenon was observed when phosphoenolpyruvate was used as the substrate for enolase activity in the absence of sorbic acid. Finally, it was demonstrated that the addition of individual amino acids to a solution containing Mg^{++} and either sorbic acid or phosphoenolpyruvate would result in a similar loss of optical density.

Mg^{++} is known to be important as an activator of enolase, and Malmstrom, Kimmel, and Smith (1959) concluded that the activation process results from a combination of the ion with the enzyme and not the substrate. Therefore, the substrate combines with a metal-enolase complex. Since these data indicate that α,β-unsaturated acids in general react or combine with enolase in the presence of Mg^{++}, it is proposed that this may be the basis of sorbic acid inhibition. At

lower levels, the inhibition may be at least partially competitive with the sorbic combining with a site(s) adjacent to the active site on the enzyme reducing the affinity of the enzyme for the substrate; whereas at higher levels, the number of sorbic acid molecules combining with each molecule of enzyme may be much greater, resulting in primarily a partially noncompetitive inhibition.

Since the report of Whitaker (1959) appeared during the course of these experiments, the work with alcohol dehydrogenase was repeated using both cell-free yeast preparations and crystalline alcohol dehydrogenase. Our data with the crystalline enzyme substantiated Whitaker's report; i.e., sorbic acid inhibited this enzyme preparation and the degree of inhibition was greatly enhanced by incubating the enzyme with sorbic acid prior to adding substrate. Whitaker observed considerable protection of the crystalline enzyme from the effects of sorbic acid by sulfur-containing amino acids. He, therefore, postulated that alcohol dehydrogenase was inhibited as a result of the formation of a stable complex between sorbic acid and the sulfhydryl groups of the enzyme. Since no inhibition of alcohol dehydrogenase activity in the yeast extracts was observed even after prolonged incubation of the extracts with sorbic acid, the concentration of protective materials in the yeast extracts must have been sufficient to offer complete protection. It is significant that yeast enolase contains no sulfhydryl groups according to Malmstrom et al. (1959); thus the enolase could not be affected in this manner.

It must be concluded from these data that the inhibition of alcoholic fermentation by sorbic acid is primarily the result of enolase inhibition. The question of the failure of this acid to affect lactic acid bacteria to any great extent is yet to be resolved. Preliminary experiments in our laboratory indicate that enolase activity in cell-free extracts of *Lactobacillus brevis* and *Pediococcus cerevisiae* is not inhibited by sorbic acid.

SUMMARY

The extent of sorbic acid inhibition of alcoholic fermentation by resting cells of baker's yeast is dependent on the pH, but no significant effect of pH was evident in the per cent inhibition of fermentation by cell-free extracts of yeast. Therefore, it is concluded that the pH effect observed with this acid is probably related to the greater permeability of the cell to the undissociated form of the acid.

Sorbic acid was shown to inhibit CO_2 production by cell-free yeast extracts from glucose, fructose 1,6-diphosphate, and 3-phosphoglyceric acid. The per cent inhibition was essentially the same with all three of these substrates. However, it did not inhibit the activity of the yeast extracts in the reduction of diphosphopyridine nucleotide with fructose 1,6-diphosphate as substrate, the oxidation of reduced diphosphopyridine nucleotide with phosphoenolpyruvate as substrate, the oxidation of reduced diphosphopyridine nucleotide with 2-phosphoglyceric acid as substrate in the presence of adenosine triphosphate and sufficient fluoride to block enolase activity, the decarboxylation of pyruvate, or the reduction of diphosphopyridine nucleotide with ethanol as substrate. Therefore, it was concluded that the site of sorbic acid inhibition was between 2-phosphoglyceric acid and phosphoenolpyruvate.

Studies with purified yeast enolase demonstrated that this enzyme was inhibited greatly by sorbic acid. The degree of inhibition was dependent on both the substrate and inhibitor concentrations; but the type of inhibition appeared to be complex. It is concluded that enolase is the primary site of sorbic acid inhibition of alcoholic fermentation.

REFERENCES

Bell, T. A., J. L. Etchells, and A. F. Borg 1959 Influence of sorbic acid on the growth of certain species of bacteria, yeasts, and filamentous fungi. J. Bacteriol., **77**, 573–580.

Costilow, R. N., W. E. Ferguson, and S. Ray 1955 Sorbic acid as a selective agent in cucumber fermentations. I. Effect of sorbic acid on microorganisms associated with cucumber fermentations. Appl. Microbiol., **3**, 341–345.

Costilow, R. N., F. M. Coughlin, E. Robbins, and Wen-tah Hsu 1957 Sorbic acid as a selective agent for cucumber fermentations. II. Effect of sorbic acid on the yeast and lactic acid fermentations in brined cucumbers. Appl. Microbiol., **5**, 373–379.

Dixon, M., and E. D. Webb 1958 *Enzymes.* Academic Press, Inc., New York.

Gooding, C. M. 1945 Process of inhibiting growth of molds. U. S. Patent 2,379,294.

Green, D. E., D. Herbert, and V. Subrahmanyan 1941 Carboxylase. J. Biol. Chem., **138**, 327–339.

MALMSTROM, B. G., J. R. KIMMEL, AND E. L. SMITH. 1959. Amino acid composition and amino-terminal sequence of yeast enolase. J. Biol. Chem., **234**, 1108–1111.

MELNICK, D., H. F. LUCKMANN, AND C. M. GOODING. 1954. Sorbic acid as a fungistatic agent for foods. VI. Metabolic degradation of sorbic acid in cheese by molds and the mechanism of mold inhibition. Food Research, **19**, 44–58.

RACKER, E. 1950. Crystalline alcohol dehydrogenase from baker's yeast. J. Biol. Chem., **184**, 313–319.

WARBURG, O., AND W. CHRISTIAN. 1941. Isolierung und Kristallisation des Gärungsferments Enolase. Biochem. Z., **310**, 384–421.

WARBURG, O., AND W. CHRISTIAN. 1943. Isolierung und Kristallisation des Gärungsferments Zymohexase. Biochem. Z., **314**, 149–176.

WHITAKER, J. R. 1959. Inhibition of sulfhydryl enzymes with sorbic acid. Food Research, **24**, 37–43.

WOLD, F., AND C. E. BALLOU. 1957. Studies on the enzyme enolase. II. Kinetic studies. J. Biol. Chem., **227**, 313–327.

YORK, G. K., AND R. H. VAUGHN. 1955. Site of microbial inhibition by sorbic acid. Bacteriol. Proc., **1955**, 20.

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[Agr. Biol. Chem., Vol. 32, No. 8, p. 940~946, 1968]

Studies on Sorbic Acid

Part IV. Inhibition of the Respiration in Yeast

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Received November 1, 1967

Sorbate inhibited the growth of baker's yeast at the logarithmic and stationary phases and its inhibition showed the Type II mode proposed by Tamiya *et al.*

Microscopic observation of the yeast cells during growth demonstrated that the sorbate inhibition was fungistatic, but not fungicidal. The respiration of the yeast was manometrically determined and the mechanism of the inhibition was suggested that sorbate would competitively combine with coenzyme A and acetate and would consequently inhibit the enzyme reaction relating coenzyme A. In addition, it was clarified that sorbyl-coenzyme A was also determined by the method of the enzymatic acetylation of sulfanilamide. This would suggest that the sorbyl moiety might be transferred to 4-amino radical of sulfanilamide enzymatically as well as in the case of acetyl-coenzyme A.

Sorbic acid and its potassium salt are widely used as food preservatives and antiseptics of medical preparations. Since these antiseptics have a broad antimicrobiological spectrum and little toxicity, they are widely applied in food.

Influence of sorbate on some enzymes has been reported in some papers. Whitaker[1] reported that sorbic acid inhibited the sulfhydryl enzymes, ficin and alcohol dehydrogenase, as a concentration of 10^{-4} M. Studies of the sorbate inhibition on alcoholic fermentation by the resting cells of baker's yeast showed that enolase is the primary site of the inhibition.[2] With the washed cells of *Escherichia coli*, *Pseudomonas aeruginosa* and *Saccharomyces cerevisiae*, York *et al.* demonstrated that the oxidative assimilation of glucose, acetate, succinate, or fumarate was inhibited by sorbic acid and that sulfhydryl enzymes such as fumarase, aspartase, and succinic dehydrogenase were the sites of inhibition.[3] Martoadiprawito[4] also reported that sorbate inhibited yeast alcohol dehydrogenase. These reports proposed that inhibition mechanism at the enzymatic level, but no report has suggested about the inhibition mechanism at the whole cell level.

Wakil[5] determined the amounts of CoA by measuring of ultraviolet absorption of sorbyl-CoA which is formed in the reaction of CoA with sorbic acid by fatty acid activating enzyme.[6] Kamihara *et al.*[7] reported that growth inhibition of *Streptococcus faecalis* 10 Cl by propionate would be due to the formation of propionyl-CoA.

Necessity should be emphasized to elucidate the physiological mechanism of inhibition at

1) J. R. Whitaker, *Food Res.*, **24**, 37 (1959).
2) J. J. Azukas, R. N. Costilow and H. L. Sadoff, *J. Bact.*, **81**, 189 (1961).
3) G. K. York and R. H. Vaughn, *J. Bact.*, **88**, 411 (1964).
4) W. Martoadiprawito and J. R. Whitaker, *Biochem. Biophys. Acta*, **77**, 536 (1963).
5) S. J. Wakil and G. Hübscher, *J. Biol. Chem.*, **235**, 1554 (1960).
6) H. R. Mahler and S. J. Wakil, *ibid.*, **204**, 453 (1953).
7) T. Kamihara, S. Yabushita, K. Yamaoka and S. Fukui, Abstracts of Papers, 18th Congress of Japan Vitamin Society, Osaka, 1966, p. 25.

whole cell level with sorbate, since, as anaerobic microorganisms are not inhibited by sorbate, it is not considered that the inhibition mechanism would be attributed only to the above mentioned sulfhydryl blocking. The authors, using baker's yeast, investigated the mode of inhibition and the influence on the respiration of sorbic acid. The results suggested that the inhibition mechanism would be of inhibition of the enzyme system relating CoA by the formation of sorbyl-CoA.

MATERIALS AND METHODS

Microorganism. *Saccharomyces cerevisiae* Oslo commercially used for bread making, was purchased from Oriental Yeast Co., Ltd. The press yeast usually contains 6.8 μg thiamine, 29.7 μg riboflavin, 10.4 μg pyridoxine, 37.9 μg pantothenic acid, 107 μg nicotinic acid, 0.14 μg biotin and 2055 μg inositol per gram by wet base.

Manometric determination. The respiration of the yeast was manometrically determined at 30°C in a total volume of 2 ml reaction mixture. The effect of initiaters, succinate or others, on the respiration of the yeast was found to be little.

Assay of CoA. Both methods of measuring ultraviolet absorption of sorbyl-CoA[5] and of enzymatic acetylation of sulfanilamide using pigeon liver as enzyme source formulated by Kaplan and Lipmann[8] were employed with some modification of the volume and the concentrations of the reaction mixture.

The reaction mixture of Wakil-Hübscher's method[5] used in this investigation contains 0.1 mmole of tris-(hydroxymethyl)-aminomethane buffer (pH 9.0), 10 μmoles of magnesium chloride, 25 μmoles of ATP, 10 μmoles of potassium sorbate and 10 μmoles of glutathione. To this mixture was added sample solution containing 1 to 50 mμmoles of CoA and the reaction was started by the addition of about 0.1 mg of fatty acid activating enzyme. The fatty acid activating enzyme was prepared by the method of Mahler and Wakil.[6] The total volume of the incubation mixture was 1 ml and a blank contained all the components but CoA or sorbate. Sometimes boiled enzyme was used for a blank.

Nitrogen gas was passed over the reaction mixture in a test tube, and the tubes were stoppered and incubated at 38°C for 10 min.

At the end of the reaction 4 ml of water was added and the optical density was measured at 300 mμ with spectrophotometer.

RESULTS

1) Growth inhibition

The maximum growth medium, containing 10% sucrose, 0.5% yeast extract powder (BACTO Yeast Extract, DIFCO), 0.3% monobasic potassium phosphate and 0.1% magnesium sulfate and sorbate at a concentration shown in Fig. 1, pH 5.2 was used to determine growth inhibition of the baker's yeast by sorbate. With aluminium caps as plugs the test tubes containing 4 ml each of the medium were sterilized at 120°C for 10 min. Incubation was carried out statically at 30°C with inoculation at over night culture at a concentration of 10^4 cells per ml.

Figure 1 showed that the growth inhibition by sorbic acid was of Type II classified by Tamiya *et al.*,[9] and of little effect in lag phase. It also indicated the significant decrease of the growth rate in the logarithmic

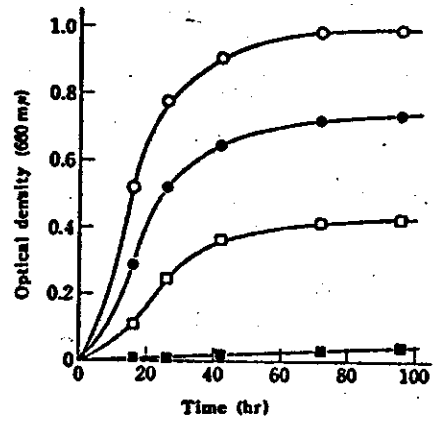

FIG. 1. Growth Inhibition of *S. cerevisiae* by Sorbate.
Sorbic acid concentration:
—○— 0%, —●— 0.012% (1.1 mM), —□— 0.048% (4.3 mM), —■— 0.191% (17 mM).

8) N. O. Kaplan and F. Lipmann, *J. Biol. Chem.*, 178, 37 (1948).

9) H. Tamiya, T. Yanagita and Y. Suzuki, *J. Penicillin*, 1, 264 (1947).

phase and of the optical density in the stationary phase. The inhibition by sorbate appeared exponential to its concentration to some extent and complete at 0.19% of sorbic acid.

2) Microscopic observation of the inhibition

The intact cells of 10^8 cells per ml of logarithmic phase were suspended to the above medium containing 0.02% methylene blue. One drop of the suspension was applied on the cover glass which was set on the hole object glass and fixed with paraffin all edges. The cell growth and the inhibition of the cell growth by sorbate was observed at 22°C under the microscope (Table I). Budding of the celll regularly needed 200 min for each time under this condition. Number of cells which were not dyed increased from 13 to 20 during 380 min. With sorbic acid the budding was no longer observed, but neither the death of cell nor formation of sporangium was recognized because no increase of number of cells which were partially dyed or dyed was observed.

3) Inhibition of respiration

Studies on the respiration of the baker's yeast have given many achievements in the field of glycolysis and bioenergetics. Inhibition mechanism of sorbic acid on the yeast respiration can be investigated by the manometric technique. The respiration of the baker's yeast was inhibited by sorbic acid as shown in Table II.

Hansen *et al.*[10] and York *et al.*[11] reported that the growth of Clostridia, anaerobic microbes, were not inhibited by sorbic, propionic or caproic acid. These facts suggest that the essential point of the inhibition mechanism may exist in the sequence of respiration. Figure 2 shows the effect of the sorbic acid concentration on Q_{O_2} or Q_{CO_2}.

TABLE II. INHIBITION OF THE YEAST RESPIRATION BY SORBIC ACID

Substrates	Control		Sorbate added*	
	Q_{O_2}	Q_{CO_2}	Q_{O_2}	Q_{CO_2}
Endogenous	13	18	7	7
Pyruvate	53	70	8	10
Glucose	78	187	61	132

Reaction mixture: Final concentration: 55 mM of each substrate, 14 mM of KH_2PO_4-HCl buffer (pH 4), 2.2 mM of $MgSO_4$ and 0.7 mg of baker's yeast by dry weight; total volume: 1.8 ml except 0.2 ml of 30% KOH in the case of measuring O_2 uptake, and in the case of CO_2 evolution 0.2 ml water was added in the center well with the aid of small crumple of filter paper. Reaction was carried out by 120 rpm shaking at 30°C in the Warburg Manometer under air gas phase.

* Sorbic acid concentration was 7.4 mM for pyruvate or 19 mM for glucose, respectively.

TABLE I. THE EFFECT OF SORBATE ON CHANGES OF THE CELL COUNTS IN THE METHYLENE BLUE (MB) TEST

Incubation time	Number of cells					
	Control			0.1% Sorbic acid added		
	Not dyed*	Partially dyed*	Dyed*	Not dyed*	Partially dyed*	Dyed*
Initial	13	3	0	13	1	1
After 110 min	14	3	0	13	0	2
After 230 min	19	0	3	13	0	2
After 380 min	20	0	3	13	0	2

The figures show the number of the cells in a definite view under the microscope. The preparation method of the specimens and the incubating conditions are mentioned in the text.

* Living cells are not dyed with MB but died cells are dyed with it. Partially dyed cells represent partially inactivated cells.

10) J. D. Hansen and M. D. Appleman, *Food Res.*, 20, 92 (1955).

11) G. K. York II and R. H. Vaughn, *Food Res.*, 20, 60 (1955).

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concentration on Q_{O_2} or Q_{CO_2}.

II. Inhibition of the Yeast
spiration by Sorbic Acid

Control		Sorbate added*	
Q_{O_2}	Q_{CO_2}	Q_{O_2}	Q_{CO_2}
13	18	7	7
53	70	8	10
78	187	61	132

ixture: Final concentration: 55 mM
rate, 14 mM of KH_2PO_4-HCl buffer (pH
$MgSO_4$ and 0.7 mg of baker's yeast
; to volume: 1.8 ml except 0.2 ml
in case of measuring O_2 uptake,
ase of CO_2 evolution 0.2 ml water was
center well with the aid of small
ilter paper. Reaction was carried out
making at 30°C in the Warburg Mano-
air gas phase.
acid concentration was 7.4 mM for
19 mM for glucose, respectively.

ES OF THE CELL
B) TEST
lls

0.1% Sorbic acid added		
Not dyed*	Partially dyed*	Dyed*
13	1	1
13	0	2
13	0	2
13	0	2

der the microscope. The preparation
med in the text.
dyed with it. Partially dyed cells

York II and R. H. Vaughn, *Food Res.*,

Table III. Influence of Sorbic Acid on the Respiration of the Different Fraction of the Disrupted Cells

Treatment of the cells	Sorbate added mM	Endogenous		Glucose as substrate		Pyruvate as substrate	
		Q_{O_2}	Q_{CO_2}	Q_{O_2}	Q_{CO_2}	Q_{O_2}	Q_{CO_2}
Whole cells	0	13	18	84	190	53	70
Whole cells	7.4	7	7	70	153	8	10
Whole cells	27.0	—	—	55	100	—	—
Cell homogenate	0	9	44	18	49	13	177
Cell homogenate	7.4	10	43	15	56	15	168
Cell homogenate	27.0	—	—	—	—	9	127
800×g supernatant of cell homogenate	0	0	3	0	3	0	66
800×g supernatant of cell homogenate	7.4	0	2	0	1	0	63
2000×g residue of cell homogenate	0	2	5	4	9	4	14
2000×g residue of cell homogenate	7.4	0	5	4	11	3	12

Reaction mixture: 55.5 mM of substrate, 11 mM of phosphate buffer pH 5.8, 2.2 mM of $MgSO_4$ at final concentration.

Destruction of the cells: Baker's press yeast was suspended in M/10 phosphate buffer pH 5.8 and treated in Nossal Shaker (same as that of Table VI). Dry matters of the enzyme sources except the whole cells were calculated from weight as the whole cells. Total reaction volume, reaction temperature and shaking rate were same as those of Table II.

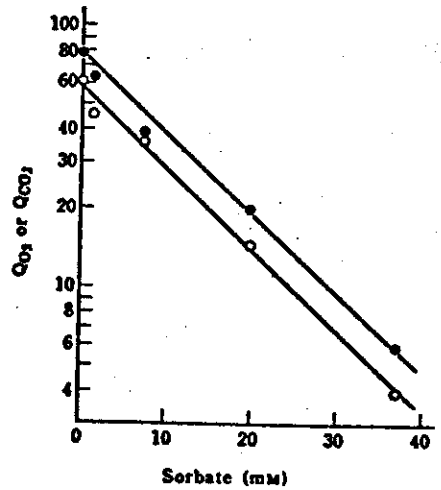

Fig. 2. Inhibition of Yeast Respiration by Sorbate.
—○— Q_{O_2}.
—●— Q_{CO_2}.
Reaction mixture was similar to that of Table II except that 55.5 mM of pyruvate and 11 mM of KH_2PO_4-HCl buffer (pH 4) were used for substrate and buffer solution, respectively.

Oxygen uptake and carbon dioxide evolution were inhibited by sorbic acid in the first order kinetics. By destruction of the cells in the Nossal Shaker, the respiration with or without sorbate markedly decreased as shown in Table III. Studies on the respiration with isolated mitochondria are under investigation.

Tables II and III showed that low inhibitory effect of sorbate on the respiration is found when glucose was used as substrate. Although fructose-1,6-diphosphate gave the similar result, the reason for these results is still obscure. However, at higher concentration of sorbate (27 mM) the respiration inhibition for glucose was found as well as those for acetate or pyruvate used as substrate. When the acids of TCA cycle were used as the substrates, Q values decreased in comparison to those for acetate or pyruvate as shown in Table IV. They are in good agreement with those reported by Krebs.[12] The inhibitory effect of sorbate on Q values decreased when the cell homogenate was used instead of the

12) H. A. Krebs, "Chemical Pathways of Metabolism," Vol. 1, ed. by David M. Greenberg, Academic Press Inc., New York, N. Y., 1954, p. 135.

TABLE IV. OXIDATION OF SOME TCA CYCLE MEMBERS BY THE YEAST

Substrates	Control		6.7 mM Sorbate added	
	Q_{O_2}	Q_{CO_2}	Q_{O_2}	Q_{CO_2}
Endogenous	8(9)	8(35)	3(5)	5(34)
Pyruvate	68(17)	73(188)	17(17)	21(190)
Acetate	97(14)	109(98)	18(13)	20(87)
Citrate	10(11)	11(41)	2(8)	3(40)
2-Oxoglutarate	6(9)	9(38)	1(9)	4(39)
Succinate	8(10)	8(38)	6(9)	8(35)
Fumarate	7(9)	9(39)	4(10)	6(38)
Malate	11(9)	11(40)	4(10)	8(40)
Oxalacetate	51(11)	58(38)	40(9)	48(36)

Reaction mixtures: 5.5~55.5 mM of substrate, other components and conditions of reaction were similar to those of Table II. The method of cell homogenization was the same as that of Table III. Figures in parentheses showed the results of Q values when the cell homogenate was used in place of the intact cells.

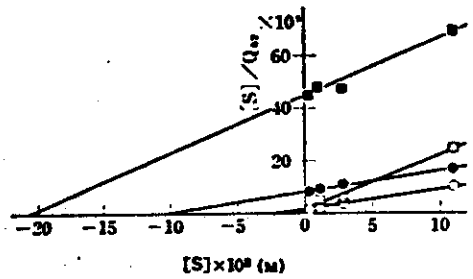

FIG. 3. Apparent *Km* for Pyruvate of Acetate and Apparent *Ki* for Sorbate.

—○— Substrate: acetate.
—●— Substrate: acetate, 3.7 mM sorbate added.
—□— Substrate: pyruvate.
—■— Substrate: pyruvate, 3.7 mM sorbate added.

Methods and conditions of reactions were similar to those of Table II.

intact cells. The sorbate inhibition on the respiration of yeast at the whole cell level was competitive with respect to pyruvate or acetate as shown in Fig. 3. In the case of pyruvate oxidation, the apparent Michaelis constant (*Km*) for pyruvate and apparent inhibition constant (*Ki*) for sorbate were determined to be 4.0×10^{-4} M and 7.2×10^{-5} M, respectively. When acetate was used as substrate, the apparent values of *Km* for acetate and *Ki* for sorbate were calculated as 1.8×10^{-3} M and 7.4×10^{-4} M, respectively.

4) Influence of addition of CoA on the sorbate inhibition and uptake of CoA by the yeast cells

It is well known that CoA has a significant role on the respiratory system. If CoA is expended with sorbate to form sorbyl-CoA, the yeast respiration may not be inhibited by sorbate when excessive CoA is added to the reaction system. Experiment was carried out under the similar conditions to those of Table II (substrate: pyruvate), the following $Q_{O_2}(Q_{CO_2})$ values were obtained: no addition, 69(91); plus 10 mM sorbate, 2(7); plus 10 μM CoA, 74(98); plus 10 mM sorbate and 10 μM CoA, 0(11); respectively [endogenous; 17(21)]. These results demonstrated that the addition of CoA had no recovery effect on the sorbate inhibition for oxidation of pyruvate by the intact cells of the baker's yeast.

This reason may be explained by the results of Table V in which no uptake of CoA into the cells was shown. The comparison of CoA contents of the incubated cells with and without CoA revealed no increase of CoA inside the cells after incubation with CoA.

The extraction method of CoA from the

TABLE V. UPTAKE OF CoA INTO THE YEAST CELLS

CoA added to the reaction mixture μg	Total amounts of CoA found after incubation	
	Cells μg	Supernatant μg
0 (Blank)	12	not detected
1058	11	1020

Reaction mixture: 1 ml of 10% suspension of the yeast by wet weight base, 1 ml of 0.1 N KH_2PO_4 (pH 5.5) and 1 ml of CoA solution (1.058 mg/ml). In case of the blank test 1 ml of water was used in place of CoA solution.

Procedure: The reaction mixture was incubated at 37°C for 1 hr and centrifuged. The cells were washed with 3 ml of water. The cells and the supernatant were applied for the assay of CoA.

cells is the same with that of Table VII.

Next experiments were performed to obtain the yeast cells containing the high content of CoA and to reveal the role of CoA in the sorbate inhibition.

The yeast was statistically cultured in a medium containing 10% glucose, 0.055% potassium phosphate (mono basic), 0.043% potassium chloride, 0.013% calcium chloride ·2aq., 0.013% magnesium sulfate·7aq., 0.00025% manganese sulfate, 0.00025% ferric chloride, 0.05% sodium citrate, 0.01% citric acid and 0.75% ammonium sulfate, pH 5.5 (control medium) or the above medium supplemented with 1 mg % calcium-D-pantothenate (pantothenate added medium) for 16 hr at 30°C.

Cells were harvested by centrifugation and washed with 0.9% sodium chloride solution. The cell cake was divided into two parts: one of them was suspended in 0.1 M phosphate buffer (pH 5.8) containing 5% sucrose and 0.01% magnesium sulfate·7aq. and incubated at 37°C for 1 hr; another part was suspended in the above solution containing 0.1% sorbic acid (pH 5.8) and incubated under the same condition. After incubation, cells were centrifuged and washed with water. The packed cells were suspended in 5 volumes of water and boiled for 5 min to extract CoA and then centrifuged. Table VI shows that this procedure was found to be the most efficient to extract CoA from the cells and that the homogenization caused the breakdown of CoA. The supernatants were assayed for CoA. Table VII showed two to three fold increase in the accumulation of CoA in the cells of the baker's yeast by the preincubation with pantothenate.

On the other hand, figures, given in parentheses in Table VII indicating the results that the reaction mixtures of the Wakil's method marked as "a" were assayed by the Kaplan's method, are larger than those obtained by Wakil's method. This fact suggests that sorbyl-CoA would be as well determined with Kaplan and Lipmann's method. This consideration could be extended to fractional determination of CoA and acyl-CoA.

TABLE VI. EXTRACTION OF CoA FROM CELLS OF BAKER'S YEAST

Extraction method	CoA μmoles/dry cell g
add 6.7 fold water and boil*1)	0.78
add 6.7 fold water, homogenize*2) and boil*1)	0.46
add 6.7 fold water, homogenize,*2) incubate*3) and boil*1)	≒0
add CoA (0.51 μmoles/dry g) with 6.7 fold water and boil*1)	1.36

*1) Mild boiling for 5 min.
*2) Cell suspension was treated in Nossal Shaker (product of B. Brawn Apparatebau.) with 0.45 to 0.5 mm diameter beads at 2000 rpm for 3 min cooling with flashing CO_2 gas from bomb.
*3) Incubated at 30°C for 30 min.

TABLE VII. AMOUNTS OF CoA IN BAKER'S YEAST

Growth medium	Sorbate	CoA found μmoles/dry g of cells	
		Wakil's method	Kaplan's method
Control	(−)	0.60	0.81
Control	(+)	0.68	0.95
Pantothenate added	(−)	1.0a	2.1 (1.5)*
Pantothenate added	(+)	1.2a	3.0 (3.0)*

* Figures given in parentheses indicate the results that the reaction mixtures of the Wakil's method marked as "a" were assayed by the Kaplan's method.

5) Recovery from sorbate inhibition of cells containing high CoA

The sorbate inhibition on the respiration of the cells containing the high content of CoA (high CoA cells) was compared with that of the ordinary cells (control cells) as shown in Fig. 4.

The fact that the respiration of the high CoA cells was little inhibited by sorbate does suggest that key substance of sorbate inhibition may be CoA.

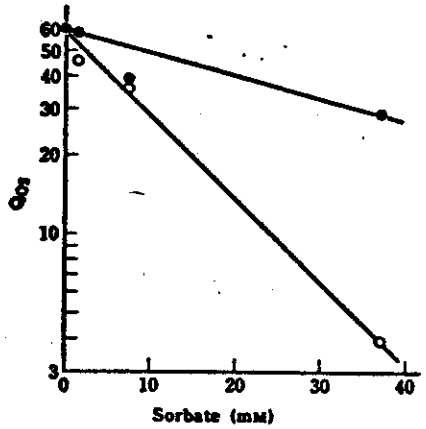

FIG. 4. Influence of Sorbate on O_2 Uptake of High CoA Cells.

—○— Control cells (0.77 μmoles CoA/dry cell g).

—●— High CoA cells (2.90 μmoles CoA/dry cell g).

DISCUSSION

The extraction procedure of CoA from the cells by the simple boiling with 5 to 10 fold volume of water was found to be the most efficient. In the case of animals, Kaplan *et al.*[8] reported little or no loss of the coenzyme by homogenizing an organ at cold. In the case of the yeast cells, however, the homogenization caused the breakdown of CoA.

Novelli and Lipmann[13] reported that the CoA contant of *S. cerevisiae* increased to three or four times by the preincubation with pantothenate under the aerobic condition. Fukui *et al.*[14] also found a significant increase approximately fifty times in the CoA level by treatment of *Saccharomyses sake* with pantothenate. In our experiment CoA content of the cells increased to two or three times by the preincubation with pantothenate.

Kaplan and Lipman's method for the CoA assay gave higher values than those by the Wakil's method. Similar results were also found by Soyama[15] in which the values obtained by the former method are approximately two times of those by the latter method. Soyama infered from his results that the assay values by Kaplan and Lipmann's method might also contain the amount of newly formed CoA from pantetheine, 4'-phosphopantetheine or dephospho-CoA during the assay procedure with the crude enzyme preparation of the pigeon liver.

It is accepted sorbate has only a slight toxicity to animals.[16] Principles of selective toxicity of sorbate have not been satisfactorily established.

Deuel *et al.*[17] reported that the intermediary metabolism of sorbate was similar to that of the normally-occurring fatty acids, such as caproic and butyric acids.

In the present paper, it was demonstrated that the sorbate inhibition in the whole cell level was competitive with pyruvate or acetate, and that the respiration of the high CoA cells was little inhibited by sorbate. As shown by Wakil *et al.*,[5] sorbyl-CoA is easily formed from CoA and sorbate by the catalytic action of fatty acid activating enzyme.[6] Kamihara *et al.*[7] indicated that the growth inhibition of *Str. faecalis* 10 Cl by propionate would be due to the formation of propionyl-CoA. These facts suggest that the sorbate inhibition of the respiration would occur by competitive action of sorbate with acetate on the site of acetyl-CoA formation.

Straight chain fatty acids are known to have antimicrobial action and the longer chain the acid has, the more inhibitory action it shows.

The plausible mechanism of the sorbate inhibition would also be applied for the inhibition by common straight chain fatty acids.

13) G. D. Novelli and F. Lipmann, *J. Biol. Chem.*, **171**, 833 (1947).
14) S. Fukui, Y. Tani and T. Kishibe, *J. Fermentation Tech.* **36**, 135 (1958).
15) K. Soyama, *Vitamins*, **34**, 209 (1966).
16) H. J. Deuel, Jr., R. Alfin-Slater, C. S. Weil and H. F. Smyth, Jr., *Food Res.*, **19**, 1 (1954).
17) H. J. Deuel, Jr., C. E. Calbert, L. Anisfeld, H. McKeehan and H. D. Blunden, *ibid.*, **19**, 13 (1954).

[VOL. 77

um linens. J. Bacteriol.,

LSO E. 1957 Growth
ococ pyogenes by man-
in. Antonie van Leeuwen-
Serol., 23, 226–234.

, R. E., AND CLARK, F. E.
reforming bacteria. Agri-
No. 16, U. S. Dept. Agr.,

ESELE, J. J. 1956 Mito-
cells: an analysis of move-
. Biochem. Cytol., 2, 319–

Synergism and potentia-
ference to combinations of
es. Pharmacol. Revs., 8,

GS, H. R. 1946 Reversal
e inhibition of *Escherichia*
ine. Arch. Biochem., 10,

45

INFLUENCE OF SORBIC ACID ON THE GROWTH OF CERTAIN SPECIES OF BACTERIA, YEASTS, AND FILAMENTOUS FUNGI[1]

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Interest in the use of sorbic acid (2,4-hexadienoic acid) as a food preservative and fungistatic agent was stimulated by Gooding in 1945 and by the report of its harmlessness as a dietary component by Deuel *et al.* (1954). The numerous reports which followed, especially those which dealt with sorbic acid as a selective inhibitor of certain microorganisms (Emard and Vaughn, 1952; York and Vaughn, 1954, 1955; Hansen and Appleman, 1955; Borg *et al.*, 1955, 1956; Costilow *et al.*, 1955) prompted this study.

The present report is concerned with the inhibitory action of sorbic acid in cultural media at different pH levels for certain species of bacteria, yeasts, and filamentous fungi which, for the most part, are associated with the growing cucumber or the commercial cucumber fermentation.

Sorbic acid falls into a group of short-chain organic acids, which, together with their salts, have been shown to exhibit antimicrobial properties. Such properties, for many of these acids, have been demonstrated for over fifty years; for reviews see: Wyss *et al.* (1945), Wyss (1948), Schelhorn (1953), Ingram *et al.* (1956), Grubb (1957).

Hoffman *et al.* (1939) investigated the fungistatic properties of normal saturated and unsaturated fatty acids containing from 1 to 14 carbon atoms and used as test organisms a mixed culture of common bread molds such as *Aspergillus niger*, *A. glaucus*, *Rhizopus nigricans*, and *Penicillium frequentans*. Sorbic acid was not included in this study. These workers reported that the effectiveness of the acids as fungistatic agents increased with chain length and concentration, and varied with the pH. Hoffman *et al.* (1941) continued this study with emphasis on the effect of pH and added to the list of chemicals tested such common food preservatives as benzoic and salicylic acids. Their work showed that these antimicrobial compounds are effective only when added in sufficient concentration to foods or culture media with a pH below 7.

Rahn and Conn (1944) compared the pH and the concentrations of undissociated benzoic and salicylic acids with the inhibitory action on *Saccharomyces ellipsoideus* and concluded that the toxic principle of such acids was the undissociated fraction and not the acid anion. As early as 1928, Rogers and Whittier reported that *Streptococcus lactis* ceased to make lactic acid when the concentration of the undissociated lactic acid reaches 0.017 M, regardless of pH. Cowles (1941) studied the action of acetic, propionic, butyric, valeric, caproic, and caprylic acids on *Staphylococcus aureus* and *Escherichia coli* and reported the bactericidal action was due to the undissociated acid fraction. Spoehr *et al.* (1949) also investigated the short-chain fatty acids as to antibacterial activity and showed a relation of activity to chain length, pH, and concentration.

Emard and Vaughn in 1952 studied the effect of sorbic acid in cultural media on microbial activity and reported that all the catalase positive cultures tested were inhibited and most of the catalase negative cultures grew without noticeable inhibition. On the other hand, these authors indicated an influence of pH on the growth of both catalase positive and negative organisms. Sorbic acid was used by Beneke and Fabian (1955) to inhibit a number of fungi isolated from tomato and strawberry fruits at pH 4.0. They also reported on the influence of sorbic acid con-

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TABLE 1

Yeasts and filamentous fungi tested for growth in basal broth A with and without 0.1 per cent sorbic acid at pH 7.0 and at pH 4.5. All cultures grew in the control basal media at both pH levels, and in the sorbic acid media at pH 7.0. All cultures were inhibited from growing in the basal media containing sorbic acid at pH 4.5

A. Yeasts

Candida	*Endomycopsis*	*Saccharomyces*
C. albicans (2, UNC)*	*E. ohmeri*	*S. cerevisiae* (2)
C. krusei	*Hansenula*	*S. fragilis* (UC)
C. tropicalis (UNC)*	*H. subpelliculosa* (2)	*S. delbrueckii*
Candida sp. A	*Pichia*	*S. lactis* (NRRL-1140)
Candida sp. B	*P. alcoholophila*	*Saccharomyces* sp.
Brettanomyces	*Pichia* sp.	*Torulaspora*
B. versatilis (2)	*Rhodotorula*	*T. rosei* (2)
Cryptococcus	*R. flava*	*Torulopsis*
C. neoformans (MH)	*R. glutinis*	*T. candida* (NRRL-211)
Cryptococcus sp. (MH)	*R. rubra* (NRRL-1592)	*T. carolineiana* (2)
Debaryomyces	*Rhodotorula* sp. A	*T. lipofera* (NRRL-1351)
D. membranaefaciens var. *Hollandicus*	*Rhodotorula* sp. B	*T. minor* (NRRL-341)
Debaryomyces sp.	*Rhodotorula* sp. C	*Zygosaccharomyces*
		Z. globiformis (2)
		Z. halomembranis

B. Filamentous Fungi

Alternaria	*Geotrichum*	*Pestalotiopsis*
A. tenuis (5)	*Geotrichum* sp. (2)	*P. macrotricha* (2)
Ascochyta	*Gliocladium*	*Phoma*
A. cucumis (4)	*G. roseum*	*Phoma* sp.
Ascochyta sp.	*Helminthosporium*	*Pullularia*
Aspergillus	*Helminthosporium* sp. (2)	*P. pullulans*
A. elegans	*Heterosporium*	*Rhizopus*
A. fumigatus (3)	*H. terrestre*	*R. arrhizus*
A. niger	*Humicola*	*R. nigricans*
A. sydowi	*H. fusco-atra*	*Rosellinia*
A. terreus	*Mucor*	*Rosellinia* sp.
A. unguis	*M. silvaticus*	*Sporotrichum*
A. versicolor (2)	*Mucor* sp. (3)	*S. pruinosum* (2)
Cephalosporium	*Myrothecium*	*Stagonospora*
Cephalosporum sp. 1 (4)	*M. roridum*	*Stagonospora* sp.
Cephalosporum sp. 2	*M. verrucaria*	*Stysanus*
Cercospora	*Myrothecium* sp.	*Stysanus* sp.
Cercospora sp.	*Papularia*	*Thielavia*
Chaetomium	*P. arundinis*	*T. basicola* (2)
C. globosum (2)	*Penicillium*	*Trichoderma*
Cladosporium	*P. atromentosum*	*T. viride* (5)
C. cladosporioides (5)	*P. chermesinum*	*Truncatella*
Colletotrichum	*P. duclauxi* (2)	*Truncatella* sp.
C. lagenarium (2)	*P. expansum* (2)	
Cunninghamella	*P. frequentans*	
C. echinulata	*P. funiculosum* (2)	
Curvularia	*P. gladioli*	
C. trifolii (2)	*P. herquei*	

TABLE 1—*Continued*

B. Filamentous Fungi

Fusarium	*P. implicatum*
F. roseum (3)	*P. janthinellum* (5)
F. oxysporum (3)	*P. oxalicum* (7)
F. solani	*P. piscarium*
F. moniliforme	*P. restrictum*
F. episphaeria	*P. rugulosum*
	P. steckii (2)
	P.-sublateritium (2)
	P. thomii
	P. urticae
	P. variabile
	Penicillium sp.

* Where more than one strain in the species was tested, this is indicated by the number in parentheses following the species. Also, the sources of yeast cultures are indicated as follows: UNC = University of North Carolina, Chapel Hill; MH = Mercy Hospital, Charlotte, North Carolina; NRRL = Northern Utilization Research and Development Division, Peoria, Illinois; UC = University of California, Davis. All other yeast cultures and all the filamentous fungi were from the authors' laboratory.

centration and pH of the media in relation to inhibition of growth.

Additional information is presented herein on the factors controlling the effectiveness of sorbic acid as a growth inhibitor for certain species of microorganisms; particular attention is given to the calculated undissociated acid concentration. A summary of part of the work has appeared earlier (Etchells *et al.*, 1955).

MATERIALS AND METHODS

Cultures tested. The filamentous fungi (table 1) represented 66 species in 32 genera. These were obtained from studies by Raymond *et al.* (1959) and Etchells *et al.* (1958) which dealt with the populations, identification, and softening enzyme activity of fungi isolated from blossoms, ovaries, and fruit of pickling cucumbers. The yeasts (table 1) represented 32 species in 12 genera and, with few exceptions, were isolated during studies on the fermentation of cucumbers (Etchells and Bell, 1950*a*, *b*; Etchells *et al.*, 1953; Borg, Etchells, and Bell, 1958, *unpublished experiments*). The lactic acid bacteria (table 3) represented 6 species in 3 genera and were obtained from the three following sources: the authors' collection, isolated from cucumber fermentations by Borg *et al.* (1956); the collection of the Dairy Manufacturing Section, North Carolina State College, Raleigh; and the collection of the Northern Utilization Research and Development Division, Peoria, Illinois.

TABLE 2

Growth of two yeast species in agar plates at pH 5.0 and pH 8.0 with different levels of sorbic acid*

pH	Total Sorbic Acid		Undissociated Sorbic Acid	*Torulopsis caroliniana*		*Saccharomyces delbrueckii*	
				Growth density†	Size of colonies	Growth density†	Size of colonies
	%	*mg/100 ml*	*mg/100 ml*				
5.0	0	0	0	5+	Large	5+	Large
5.0	0.05	50	18	3+	Medium	5+	Large
5.0	0.10	100	37	—	None	2+	Small
8.0	0	0	0	5+	Large	5+	Large
8.0	0.05	50	0	4+	Large	5+	Large
8.0	0.10	100	0	3+	Large	5+	Large
8.0	1.00	1000	1	2+	Small	2+	Small
8.0	2.00	2000	2	2+	Small	1+	Small

* Broth A with 2.0 per cent agar.

† Growth density of colonies on agar plates; 5+ = extremely heavy; 4+ = moderately heavy; 3+ = heavy; 2+ = moderately light; 1+ = light; — = no growth.

Media. The stock cultures were maintained, with frequent transfers, on the following solid media: potato-dextrose agar slants for the filamentous fungi; vegetable-juice agar slants (Etchells and Bell, 1950*a*) for the yeasts; and deep liver agar stabs (Haynes *et al.*, 1955) for the lactic acid bacteria. Growth tests were made

TABLE 3

Influence of 0.1 per cent sorbic acid at different pH levels on the growth of certain lactic acid bacteria and yeasts

Organism Tested	Growth with 0.1% Sorbic Acid* at pH						
	6.8	6.0	5.5	5.0	4.5	4.0	3.5
Bacteria							
Lactobacillus							
L. brevis (7)†	+	+	+	+	+	+	–
L. brevis (NCS)‡	+	+	+	+	+	–	–
L. plantarum (7)†	+	+	+	+	+	+	–
L. plantarum (NRRL-531)	+	+	+	+	+	+	–
L. arabinosus (NCS)	+	+	+	+	–	–	–
L. fermenti (NCS)	+	+	+	+	–	–	–
Pediococcus							
P. cerevisiae (19)†	+	+	+	+	+	–	–
P. cerevisiae (NRRL-1325)	+	+	+	+	+	–	–
Streptococcus							
S. lactis (NCS)	+	+	+	+	–	–	–
Yeasts							
Candida							
C. albicans	+	+	+	–	–	–	–
C. krusei	+	+	+	+	–	–	–
Brettanomyces							
B. versatilis	+	+	+	–	–	–	–
Cryptococcus							
C. neoformans	+	–	–	–	–	–	–
Debaryomyces							
D. membranaefaciens var. *Hollandicus*	+	+	–	–	–	–	–
Endomycopsis							
E. ohmeri	+	+	–	–	–	–	–
Hansenula							
H. subpelliculosa	+	+	–	–	–	–	–
Rhodotorula							
R. glutinis	+	–	–	–	–	–	–
Saccharomyces							
S. fragilis	+	+	+	–	–	–	–
S. delbrueckii	+	+	+	–	–	–	–
Torulaspora							
T. rosei	+	+	+	–	–	–	–
Torulopsis							
T. caroliniana	+	+	+	–	–	–	–
Zygosaccharomyces							
Z. globiformis (No. 29)	+	+	+	+	–	–	–
Z. globiformis (No. 466)	+	+	+	–	–	–	–
Z. halomembranis	+	+	–	–	–	–	–

* All organisms listed grew in the control broths.

† Indicates number of cultures tested from cucumber brines; in all other cases, single isolates were tested.

‡ NCS refers to cultures obtained from Dairy Manufacturing Section, North Carolina State College.

in basal broths with and without sorbic acid (Carbide and Carbon Chemicals Corp., New York). In one test, an agar medium was used and is so indicated. The three broths used were as follows: broth A, for testing fungi and yeasts consisted of 0.5 per cent peptone (Difco), 0.25 per cent yeast extract (Difco), 0.5 per cent sodium chloride, and 2.0 per cent glucose; broth B, for yeasts (Wickerham, 1951) contained 0.67 per cent yeast nitrogen-base (Difco), 0.1 per cent yeast extract, and 2.0 per cent glucose; broth C, for lactic acid bacteria was that recommended by Speck (1955, *personal communication*) and was composed of 1.5 per cent trypticase-soy (BBL), 0.5 per cent phytone (BBL), 0.1 per cent sodium citrate, 0.4 per cent sodium chloride, and 0.02 per cent L-cystine (dissolved first in a few drops of 2 N HCl). The basal broths with and without sorbic acid were adjusted to the desired pH values with 6 N HCl or 2.5 N NaOH using a model H-2, Beckman pH meter. Ten-ml amounts of the broths in 18 mm by 150 mm culture tubes were autoclaved at 15 psi for 15 min.

Inoculation and measurement of growth. For the yeasts and bacteria, one drop per tube from a 24- to 72-hr broth culture, grown in the corresponding medium under test, was used as inoculum. The cultures were incubated at 30 C and their growth measured as optical density at 1, 3, 7, and 14 days using a Lumetron colorimeter with 650 mμ filter. For the fungi, bits of mycelia from 7 to 14-day old potato-dextrose agar slants were transferred directly to the surface of the broths. The fungi were incubated at 30 C and the growths (mycelial mats) were observed visually at 5, 15, and 30 days.

RESULTS

Effect of sorbic acid concentration at pH 5.0 and pH 8.0. Two yeasts, *Torulopsis caroliniana* and *Saccharomyces delbrueckii*, were selected for testing the influence of sorbic acid concentration on growth at two widely separated pH levels. These yeasts are associated with the early, rapid fermentation of brined cucumbers (Etchells and Bell, 1950*a*; Etchells *et al.*, 1952) and have the property of growing over a wide pH range. The two yeast species were grown in basal broth A for about 72 hr, then 1 ml of a 1:1000 dilution was seeded into agar containing two levels of sorbic acid and at pH 5.0, and four levels of the acid at pH 8. The results of these agar plate tests are given in table 2. At pH 5.0, *T. caroliniana* was

completely inhibited with
acid; with 0.05 per cent there
but the colonies were restri
brueckii was not inhibited a
restriction in colony size wa
per cent acid. At pH 8.0, th
were only slightly inhibited
centrations from 0.05 to 2.0
there was definite restriction
It is probable that this chem
trations at pH 8.0 has little o
on these two organisms. The
growth observed was probab
in pH of the medium toward
by growth of the test yeasts.
would increase the effectivene
as an inhibitor.

Screening test on yeasts an
172 cultures of yeasts and fun
inoculated into the following
broth A with 0.1 per cent sor
(b) broth A, control, at pH 4.
0.1 per cent sorbic acid, at pH
control, at pH 7.0. The yeas
fungi grew well in the control
values. In the sorbic acid br
ganisms tested grew equally
conversely, none grew at pH

Results of experiments co
various pH levels on the grow
erties of sorbic acid for certai
acid bacteria and yeasts in liq
sented in table 3. Additional
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Lactic acid bacteria. Thirty-
resenting 6 species in 3 gener
the sorbic acid tests. Seven
Lactobacillus brevis and *L. p*
cultures of *Pediococcus cerevisia*
commercial cucumber fermen
cluded in the tests because of
(Phillips and Mundt, 1950; E
1952; Borg *et al.*, 1955; Cost
1956, 1957) on the effect of s
growth of these species, both
mentations and in selective m

The lactic acid bacteria grew
0.1 per cent sorbic acid at the
i. e., pH 5.0 and above. No
tested grew at pH 3.5 with 0.
acid; only two species, *L. brevis*
grew at pH 4.0; *P. cerevisiae* (
at pH 4.5. *L. arabinosus*, *L. ferm*

completely inhibited with 0.1 per cent sorbic acid; with 0.05 per cent there was definite growth but the colonies were restricted in size. *S. delbrueckii* was not inhibited at pH 5.0, but some restriction in colony size was observed with 0.1 per cent acid. At pH 8.0, the two yeast species were only slightly inhibited in sorbic acid concentrations from 0.05 to 2.00 per cent; however, there was definite restriction in colonial growth. It is probable that this chemical in high concentrations at pH 8.0 has little or no inhibiting effect on these two organisms. The restriction of colony growth observed was probably caused by a shift in pH of the medium toward the acid side caused by growth of the test yeasts. Such a pH change would increase the effectiveness of the sorbic acid as an inhibitor.

Screening test on yeasts and higher fungi. The 172 cultures of yeasts and fungi (see table 1) were inoculated into the following 4 test broths: (a) broth A with 0.1 per cent sorbic acid, at pH 4.5 (b) broth A, control, at pH 4.5; (c) broth A with 0.1 per cent sorbic acid, at pH 7.0; (d) broth A, control, at pH 7.0. The yeasts and filamentous fungi grew well in the control broths at both pH values. In the sorbic acid broths, all of the organisms tested grew equally well at pH 7.0 and conversely, none grew at pH 4.5.

Results of experiments on the influence of various pH levels on the growth-inhibitory properties of sorbic acid for certain species of lactic acid bacteria and yeasts in liquid media are presented in table 3. Additional information pertinent to this portion of the study follows.

Lactic acid bacteria. Thirty-nine cultures, representing 6 species in 3 genera were selected for the sorbic acid tests. Seven cultures each of *Lactobacillus brevis* and *L. plantarum*, and 19 cultures of *Pediococcus cerevisiae*, all isolated from commercial cucumber fermentations, were included in the tests because of conflicting reports (Phillips and Mundt, 1950; Emard and Vaughn, 1952; Borg *et al.*, 1955; Costilow *et al.*, 1955, 1956, 1957) on the effect of sorbic acid on the growth of these species, both in cucumber fermentations and in selective media studies.

The lactic acid bacteria grew in broth C with 0.1 per cent sorbic acid at the higher pH levels, i. e., pH 5.0 and above. None of the species tested grew at pH 3.5 with 0.1 per cent sorbic acid; only two species, *L. brevis* and *L. plantarum* grew at pH 4.0; *P. cerevisiae* (20 cultures) grew at pH 4.5. *L. arabinosus*, *L. fermenti*, and *S. lactis* were the most susceptible of the acid-forming bacteria tested and did not grow below pH 5.0 in the broth containing 0.1 per cent sorbic acid.

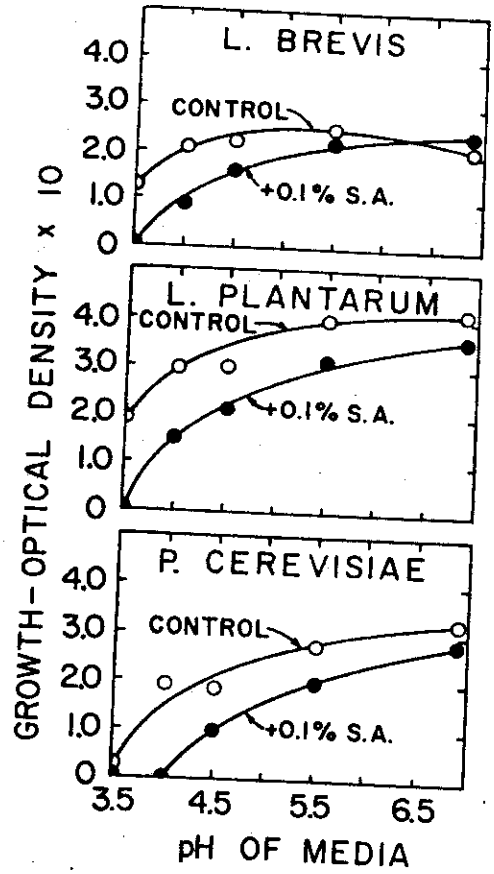

Figure 1. Effect of sorbic acid (S.A.) and pH of the broth media on growth of lactic acid bacteria isolated from commercial cucumber fermentations. *Top: Lactobacillus brevis*, average of 7 cultures. *Middle: Lactobacillus plantarum*, average of 7 cultures. *Bottom: Pediococcus cerevisiae*, average of 19 cultures. Growth measured at 14 days.

The cell growths as measured by optical density of the broth cultures were recorded for those organisms listed in table 3. Growth curves for the three predominant species of lactic acid bacteria from commercial cucumber fermentations, namely, *L. brevis*, *L. plantarum*, and *P. cerevisiae*, are shown in figure 1. In the presence of 0.1 per cent sorbic acid, a definite and increasing inhibition was observed with the above species over the pH range from 6.8 to 3.5. There was one exception however, *L. brevis* at pH 6.8; this species grew better in the sorbic acid broth at this pH level than in the control.

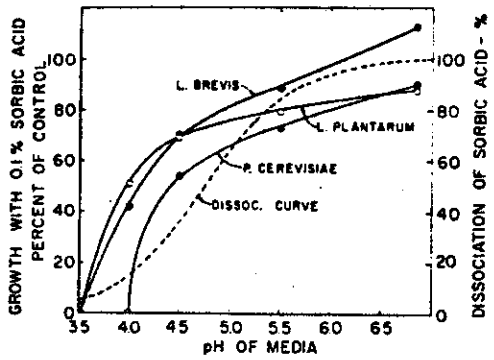

Figure 2. Growth of 3 species of lactic acid bacteria in sorbic acid broth, expressed as per cent of control, and the calculated dissociation curve of sorbic acid plotted from pH 3.5 to 6.8.

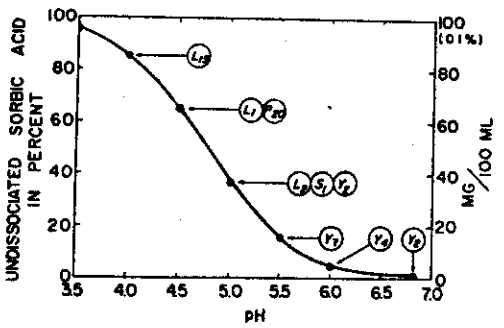

Figure 3. The calculated undissociated sorbic acid, shown in per cent and mg per 100 ml of 0.1 per cent solution, is drawn from pH 3.5 to 6.8. The lowest pH values where growth was observed for the organisms tested are indicated on the curve in the circles by *L* for *Lactobacillus* spp., *P* for *Pediococcus* sp., *S* for *Streptococcus* sp., and *Y* for yeast spp. The number of cultures tested is also indicated in the circles. See table 3 for names of organisms tested.

Yeasts. Sixteen cultures representing 14 species of yeasts were tested in broth B with 0.1 per cent sorbic acid at seven pH levels from 3.5 to 6.8 (table 3). All of the cultures grew at pH 6.8, 14 of them at pH 6.0, 10 at pH 5.5, 2 at pH 5.0, and none at pH 4.5 and below. The 2 species most tolerant to sorbic acid were *Candida krusei* and *Zygosaccharomyces globiformis*; the 2 most susceptible species were *Rhodotorula glutinis* and the pathogen, *Cryptococcus neoformans*. All yeast cultures grew in the control broths without sorbic acid at all pH levels employed.

Relationship of undissociated sorbic acid concentration to antimicrobial activity. When the growth data for three species of lactic acid bacteria (*L. brevis*, *L. plantarum*, and *P. cerevisiae*) in sorbic acid broth are plotted with the dissociation curve for the acid from pH 3.5 to 7.0, the reduction in growth of these species parallels the dissociation of the acid (figure 2). Earlier evidence of this was presented in table 2 for the yeast *T. caroliniana*. The toxic action of sorbic acid correlates with the concentration of undissociated acid. As indicated earlier in this paper, this relationship between undissociated acid and antimicrobial activity has been reported by investigators a number of times with other organic acids used as fungistatic agents.

The concentration of undissociated sorbic acid required to inhibit growth for certain of the organisms tested, may be derived from the results given in table 3. Growth of some of the organisms at the lowest pH value is indicated on the undissociated curve presented in figure 3. As an example, there were 15 Lactobacilli cultures which grew in broth media containing 0.1 per cent sorbic acid (100 mg per 100 ml) at pH 4.0 but did not grow at pH 3.5. At pH 4.0, 85 per cent of the sorbic acid (85 mg per 100 ml) is undissociated; this amount permitted the growth of the 15 cultures. At pH 3.5, 95 per cent of the sorbic acid (95 mg per 100 ml) is undissociated; this amount inhibited growth.

Further observation of the undissociated sorbic acid curve (figure 3) with respect to yeast growth reveals that only two cultures grew at pH 5.0 with 37 per cent undissociated acid; 14 yeast cultures were inhibited with this level of the acid. A good separation zone on the curve to inhibit the yeasts, yet permit good growth of the lactic acid bacteria, is about 30 to 40 mg per 100 ml of undissociated sorbic acid.

DISCUSSION

The efficiency of sorbic acid as a selective inhibitor of certain microorganisms or as a food preservative would have to be based upon the undissociated acid concentration and not the total concentration. A suggested efficiency pattern of sorbic acid for microbial inhibition, based upon increased hydrogen ion concentration, is as shown in table 4. As shown in the table, a drop in pH from 5.3 to 4.3 causes a threefold increase in the percentage of undissociated sorbic acid.

It has been shown herein that 0.1 per cent sorbic acid did not inhibit the growth of certain species of fungi, yeasts, and lactic acid bacteria

TAB

Efficiency pattern of so
inhibition based upon
concen

pH	Undissociated Sorbic Acid
	%
7.0	1
6.0	5
5.3	25
5.1	33
4.8	50
4.3	75
3.8	90
3.1	99

at pH 7. From a search
parent that sorbic acid
fatty acids are not ef
agents at neutral pH va
Vaughn (1954, 1955) an
(1955) studied the actio
media at such pH levels
Clostridium and reported
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Jacobs (1940) and An
on cell permeability to
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that pass through the
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phosphate uptake, acetat
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more at pH 4.0 than at
findings, it seems prob
property of sorbic acid i
pH because the acid is p
in the undissociated for
side the cell, the mechan
tion is not understood.
(1954) have suggested
hibits dehydrogenation

SUM

The influence of sorbi
cultures, representing 6
fungi, 32 species of yeast
acid bacteria cultures, w
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with the growing cucu
cucumber fermentation.

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TABLE 4

Efficiency pattern of sorbic acid for microbial inhibition based upon increased hydrogen ion concentration

pH	Undissociated Sorbic Acid	Antimicrobial Efficiency
	%	
7.0	1	None
6.0	5	
5.3	25	Minimal range
5.1	33	
4.8	50	
4.3	75	
3.8	90	Maximal range
3.1	99	

at pH 7. From a search of the literature it is apparent that sorbic acid and other short chain fatty acids are not effective as antimicrobial agents at neutral pH values or above. York and Vaughn (1954, 1955) and Hansen and Appleman (1955) studied the action of sorbic acid in culture media at such pH levels using certain species of *Clostridium* and reported no inhibition of growth but rather, a utilization of the acid as a carbon source.

Jacobs (1940) and Anderson (1945), in studies on cell permeability to weak electrolytes, have pointed out that it is the undissociated molecules that pass through the cell membranes. Samson *et al.* (1955) have demonstrated that, based upon phosphate uptake; acetate (0.2 M) inhibits glucose fermentation by *Saccharomyces cerevisiae* much more at pH 4.0 than at pH 7.0. In view of these findings, it seems probable that the inhibitory property of sorbic acid increases with decreasing pH because the acid is permeable to the cell only in the undissociated form of the latter. Once inside the cell, the mechanism of sorbic acid inhibition is not understood, although Melnick *et al.* (1954) have suggested that this chemical inhibits dehydrogenation of fatty acids.

SUMMARY

The influence of sorbic acid on the growth of cultures, representing 66 species of filamentous fungi, 32 species of yeasts, and 6 species of lactic acid bacteria cultures, was studied. These microorganisms, for the most part, were associated with the growing cucumber or the commercial cucumber fermentation.

The pH of the culture medium was found to be the principal factor controlling the effectiveness of sorbic acid as an inhibitor for microbial growth. All of the organisms studied grew in media containing 0.1 per cent sorbic acid at pH 7.0. The yeasts and filamentous fungi were inhibited in media containing 0.1 per cent sorbic acid at pH 4.5; the lactic acid bacteria were inhibited at this concentration of the chemical at pH 3.5.

Certain species of yeasts and lactic acid bacteria were studied in media with 0.1 per cent sorbic acid over a pH range from 3.5 to 6.8. The reduction in growth of these organisms paralleled the dissociation of sorbic acid over the pH range studied. Thus, the toxic action of sorbic acid appears to be directly related to the concentration of undissociated acid.

The efficient use of sorbic acid as an antimicrobial agent would be at a pH value which resulted in the highest concentration of undissociated acid. At pH 4.8, 50 per cent of the acid is undissociated; thus, at this pH or below, one half of the total sorbic acid or more would be effective.

REFERENCES

Anderson, E. H. 1945 Studies on the metabolism of the colorless alga, *Prototheca zopfii*. J. Gen. Physiol., **28**, 297–327.

Beneke, E. S. and Fabian, F. W. 1955 Sorbic acid as a fungistatic agent at different pH levels for molds isolated from strawberries and tomatoes. Food Technol., **9**, 486–488.

Borg, A. F., Etchells, J. L., and Bell, T. A. 1955 The influence of sorbic acid on microbial activity in commercial cucumber fermentations. Bacteriol. Proc., **1955**, 19.

Borg, A. F., Etchells, J. L., and Bell, T. A. 1956 Bloater formation by gas-forming lactic acid bacteria in cucumber fermentations. Bacteriol. Proc., **1956**, 28.

Costilow, R. N., Ferguson, W. E., and Ray, S. 1955 Sorbic acid as a selective agent in cucumber fermentations. I. Effect of sorbic acid on microorganisms associated with cucumber fermentations. Appl. Microbiol., **3**, 341–345.

Costilow, R. N., Coughlin, F. M., Robach, D. L., and Ragheb, H. S. 1956 A study of the acid-forming bacteria from cucumber fermentations in Michigan. Food Research, **21**, 27–33.

Costilow, R. N., Coughlin, F. M., Robbins, E. K., and Hsu, Wen-Tah 1957 Sorbic acid as a selective agent in cucumber fermentations. II. Effect of sorbic acid on the yeast and lactic acid fermentations in brined cucumbers. Appl. Microbiol., **5**, 373–379.

COWLES, P. B. 1941 The germicidal action of the hydrogen ion and of the lower fatty acids. Yale J. Biol. Med., **13**, 571–578.

DEUEL, H. J., JR., ALFIN-SLATER, R., WEIL, C. S., AND SMYTH, H. F., JR. 1954 Sorbic acid as a fungistatic agent for foods. I. Harmlessness of sorbic acid as a dietary component. Food Research, **19**, 1–12.

EMARD, L. O. AND VAUGHN, R. H. 1952 Selectivity of sorbic acid media for the catalase negative lactic acid bacteria and clostridia. J. Bacteriol., **63**, 487–494.

ETCHELLS, J. L. AND BELL, T. A. 1950*a* Film yeasts on commercial cucumber brines. Food Technol., **4**, 77–83.

ETCHELLS, J. L. AND BELL, T. A. 1950*b* Classification of yeasts from the fermentation of commercially brined cucumbers. Farlowia, **4**, 87–112.

ETCHELLS, J. L., BELL, T. A., AND JONES, I. D. 1953 Morphology and pigmentation of certain yeasts from brines and the cucumber plant. Farlowia, **4**, 265–304.

ETCHELLS, J. L., BELL, T. A., AND BORG, A. F. 1955 The influence of sorbic acid on the growth of certain species of yeasts, molds, and bacteria. Bacteriol. Proc., **1955**, 19–20.

ETCHELLS, J. L., BELL, T. A., MONROE, R. J., MASLEY, P. M., AND DEMAIN, A. L. 1958 Populations and softening enzyme activity of filamentous fungi on flowers, ovaries, and fruit of pickling cucumbers. Appl. Microbiol., **6**, 427–440.

ETCHELLS, J. L., COSTILOW, R. N., AND BELL, T. A. 1952 Identification of yeasts from commercial cucumber-fermentations in northern brining areas. Farlowia, **4**, 249–264.

GOODING, C. M. (to the Best Foods, Inc.) 1945 Process of inhibiting growth of molds. U. S. Patent, 2,379,294.

GRUBB, T. C. 1957 Symposium on antimicrobial preservatives. Bacteriol. Revs., **21**, 251–254.

HANSEN, J. D. AND APPLEMAN, M. D. 1955 The effect of sorbic, propionic, and caproic acids on the growth of certain clostridia. Food Research, **20**, 92–96.

HAYNES, W. C., WICKERHAM, L. J., AND HESSELTINE, C. W. 1955 Maintenance of cultures of industrially important microorganisms. Appl. Microbiol., **3**, 361–368.

HOFFMAN, C., SCHWEITZER, T. R., AND DALBY, G. 1939 Fungistatic properties of the fatty acids and possible biochemical significance. Food Research, **4**, 539–545.

HOFFMAN, C., SCHWEITZER, T. R., AND DALBY, G. 1941 Fungistatic properties of antiseptics and related compounds; effect of pH. Ind. Eng. Chem., **33**, 749–751.

INGRAM, M., OTTAWAY, F. J. H., AND COPPOCK, J. B. M. 1956 The preservative action of acid substances in food. Chem. and Ind. (London), No. **42**, 1154–1163.

JACOBS, M. H. 1940 Some aspects of cell permeability to weak electrolytes. Cold Spring Harbor Symposia Quant. Biol., **8**, 30–39.

MELNICK, D., LUCKMANN, F. H., AND GOODING, C. M. 1954 Sorbic acid as a fungistatic agent for foods. VI. Metabolic degradation of sorbic acid in cheese by molds and the mechanism of mold inhibition. Food Research, **19**, 44–58.

PHILLIPS, G. F. AND MUNDT, J. O. 1950 Sorbic acid as inhibitor of scum yeast in cucumber fermentations. Food Technol., **4**, 291–293.

RAHN, O. AND CONN, J. E. 1944 Effect of increase in acidity on antiseptic efficiency. Ind. Eng. Chem., **36**, 185–187.

RAYMOND, F. L., ETCHELLS, J. L., BELL, T. A., AND MASLEY, P. M. 1959 Filamentous fungi from blossoms, ovaries and fruit of pickling cucumbers. Mycologia (*accepted for publication*).

ROGERS, L. A. AND WHITTIER, E. O. 1928 Limiting factors in lactic fermentation. J. Bacteriol., **16**, 211–229.

SAMSON, F. E., KATZ, A. M., AND HARRIS, D. L. 1955 Effects of acetate and other short-chain fatty acids on yeast metabolism. Arch. Biochem. Biophys., **54**, 406–423.

SCHELHORN, M. VON 1953 Efficacy and specificity of chemical food preservatives. Food Technol., **7**, 97–101.

SPOEHR, H. A., SMITH, J. H. C., STRAIN, H. H., MILNER, H. W., AND HARDIN, G. J. 1949 Fatty acid antibacterials from plants. Carnegie Inst. of Washington Pub. No. **586**, 67 pp.

WICKERHAM, L. J. 1951 Taxonomy of yeasts. U. S. Dept. Agr. Tech. Bull. No. **1029**, 56 pp.

WYSS, O. 1948 Microbial inhibition by food preservatives. Advances in Food Research, **1**, 373–393.

WYSS, O., LUDWIG, B. J., AND JOINER, R. R. 1945 The fungistatic and fungicidal action of fatty acids and related compounds. Arch. Biochem., **7**, 415–425.

YORK, G. K., II, AND VAUGHN, R. H. 1954 Use of sorbic acid enrichment media for species of *Clostridium*. J. Bacteriol., **68**, 739–744.

YORK, G. K., II, AND VAUGHN, R. H. 1955 Resistance of *Clostridium parabotulinum* to sorbic acid. Food Research, **20**, 60–65.

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