Monograph on Agar-Agar 12/1/72 FDA # 72-104

MONOGRAPH
ON
AGAR - AGAR

TR-72-1552-12

Submitted Under:
Contract No. FDA 72-104

December 1, 1972

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AGAR-AGAR

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AGAR-AGAR

Summary

Agar was discovered by a Japanese innkeeper about 1658 and was first introduced to Europe and the United States from China in the nineteenth century. Its use as a laxative was first proposed by A. Schmidt in 1905 and in recent years it has enjoyed wide use as a gelling agent in the food industry, resulting in considerable interest in its physiological effects.

Booth et al. (28) reported that agar has a digestibility of 21% when fed in the diet of rats at a level of 15%, while Nilson and Schaller (193) determined the digestibility to be 28% irrespective of the level of agar consumed.

Studies by De and Guha indicate that agar is a favorable medium for the growth of intestinal microflora (54).

A depression of phagocytosis of Shigella dysenteriae was reported by Olitzki and Koch following the intraabdominal injection of agar in mice (198).

Ingestion of agar reduces the apparent digestibility of the dry matter of the diet - 10% depression at 30% agar (193).

Meuser et al. (178) reported that agar causes the release of histamine from in vitro rabbit thrombocytes.

Polynucleotide kinase was found by Wu to be strongly inhibited in the presence of agar (291).

The inhibition of Staphylococcus aureus by polymyxin, neomycin, kanamycin sulfate and streptomycin sulfate was found by Hanus et al. to be adversely affected by the presence of agar (109).

Inhibition of Micrococcus pyogenes var. aureus by chlortetracycline, chloramphenicol and penicillin was augmented by agar while the inhibition by streptomycin was reversed (134).

No determinations of the acute toxicity of agar have been reported, but a number of short-term feeding studies have been published.

Vohra and Kratzer found no effect of agar on the growth rate of day-old Arbor-Acres chicks when fed ad lib at a level of 2% of the diet for a period of 20 days (279).

Nilson and Schaller found that rats given 10% agar in their diet gained weight about 20% faster than control animals. They also noted that rats fed 20 and 30% agar diets required significantly more water per gram gain in weight than did the controls (193).

A slight depression of growth rate was noted by Ershoff and McWilliams when rats were fed 10% agar. It was suggested that this may be due in part to the great affinity of agar for water, resulting in a decrease in the absorption and utilization of water-soluble nutrients (67).

A significant (18%) reduction in food efficiency, but not weight gain, was recorded by Booth et al. during a 62-day feeding study in which 6 weanling male rats received a 15% agar diet (28).

Although no effect on body weight was observed, definite increases in the length of the colon and cecum and in the surface area of the small intestine and the colon (excluding cecum) were noted by Wierda in rats fed 30% agar for 40 weeks. No such effects were found in rats given 10% agar for 30 weeks (284).

Fifty weanling albino rats maintained by Wierda for 44 weeks on a diet containing 30% agar showed no difference in body weight from the controls, but exhibited an average weight of the large intestine 80% greater than the controls (285).

Following the feeding of 25% agar diets to 6 weanling male rats for a period of 4 weeks, Fischer found significant increases in the dry weight of the stomach, the cecum, and the colon plus rectum. During the 3rd week of feeding, a reduction in weight gain was noted for the agar-fed rats (73).

On the basis of an experiment in which a group of 6 rats was fed 10% agar for 82-107 days, Schulz and Thomas concluded that agar probably induced the deposition of fats having lower iodine values than the control animals (231).

Schulz and Thomas reported that the addition of 10% agar to the diet of 6 rats had no effect on the percentage retention of lipids (232).

Feeding studies by Hove and Herndon indicated that a 20% dietary supplement of agar, fed for 40 days, favorably influenced the growth rate of rabbits (123).

Agar has been used as a laxative, in daily doses from 4 to 15 g, since 1905. The efficiency of agar is not great, with the effect usually not noticeable for nearly a week (250).

Investigations by Frohberg et al. disclosed that the intraperitoneal injection of 0.2 ml of a 1% agar solution daily from the 4th to the 8th day of gestation in NMRI mice resulted in a somewhat higher percentage of resorptions in the agar-injected mice than in the controls (82).

AGAR-AGAR

Chemical Information

I. Nomenclature

A. Common Names
1. Gelose
2. Japan agar
3. Bengal isinglass
4. Ceylon isinglass
5. Chinese isinglass
6. Japan isinglass
7. Vegetable isinglass
8. Seaweed isinglass
9. Japanese gelatin
10. Bar kanten
11. Square kanten
12. Slender kanten
13. Tungfen

B. Chemical Names
A complex polysaccharide of galactose

C. Trade Names
Laylor Carang

D. Chemical Abstracts Registry No.
PM9002180

II. Empirical Formula

Not applicable

III. Structural Formula

Originally, agar was thought to consist of two polysaccharides, agarose and agaropectin. Agarose was believed to be essentially neutral and agaropectin was believed to be highly charged with acidic groups (61).

According to Duckworth and Yaphe (61), agar is a complex polysaccharide consisting of chains having alternating alpha (1→3) and beta (1→4) linkages. The total charge content varies for each species, time of year, and stage of growth in the plant. The extremes of the structure are as follows:

a. Neutral agarose. The idealized structure of a molecule with alternating (1→4)-linked 3,6-anhydro-alpha-L-galactose and (1→3)-linked beta-D-galactose residue containing no charge groups.

b. Pyruvated agarose with little sulphation. The D-galactose residues are substituted by 4,6-O-(1-carboxyethylidene)-D-galactose residues until the substitution reaches one in twenty. At this point the concentration of sulphate is 2%, indicative of substitution of the 3,6-anhydro-alpha-L-galactose residues with galactose sulphate.

c. Sulphated galactan containing no or little 3,6-anhydro-L-galactose or 4,6-O-(1-carboxyethylidene-D-galactose residues. The substitution of the D-galactose residues by 4,6-O-(1-carboxyethylidene)-D-galactose residues gradually decreases and the concentration of galactose sulphate increases to a non-gelling sulphated glycan (61).

Izumi suggests that it is necessary to reconsider the nomenclature of agar. Agarose may be retained for the essentially neutral polysaccharide consisting of repeated 4-O-beta-D-galactopyranosyl-3-6-anhydro-L-galactopyranose residue. Agaropectin should be discontinued since its original meaning has become ambiguous (136).

IV. Molecular Weight

The molecular weight of agar has been estimated to be between 5,000-30,000. Dental-type flake agar may be as high as 110,000 and laboratory extracted agars may be as high as 160,000 (235).

V. Specifications

C. Food Chemicals Codex

Water absorption	Passes test
Limits of Impurities	
Arsenic (as As)	Not more than 3 ppm (0.0003%)
Ash (Total)	Not more than 6.5% on the dried basis
Ash (acid-insoluble)	Not more than 0.5% on the dried basis
Gelatin	Passes test
Heavy metals (as Pb)	Not more than 40 ppm (0.004%)
Insoluble matter	Not more than 1%
Lead	Not more than 10 ppm (0.001%)
Loss on drying	Not more than 20%
Starch	Passes test

VI. Description

A. General Characteristics

1. white to pale yellow color
2. odorless or slight characteristic odor
3. tasteless or mucilaginous taste
4. strips, coarse, or fine powder

B. Physical Properties

1. melting point of gel 30-97 deg. C, depending on source
2. elastic
3. insoluble in cold water or alcohol
4. slowly soluble in boiling water, formamide, and slightly soluble in ethanolamine
5. a 1.5% aqueous soln. should be clear, and forms a resilient gel when cooled to 32-39 deg C.
6. exhibits synersis
7. colloid character

C. Stability

Dry agar deteriorates upon storage. Processing changes which reduce sulfate and nitrogen appear to increase stability.

VII. Analytical Methods

Agar-agar occurs in many commercial products. The official method for the determination of agar in meat is to treat the sample with trichloroacetic acid and centrifuge to precipitate the gum (14).

When the gum is isolated, it can be determined qualitatively by a systematic analytical scheme such as one devised by Ewart and Chapman (68). This method is based on precipitation reactions with calcium chloride, sodium hydroxide, barium hydroxide, and lead acetate. This scheme distinguishes between agar, pectin, alginate, gelatin, starch, carboxymethylcellulose, methylcellulose, and several other gums, including carrageenan, tragacanth, locust, karaya, ghatti. The same gums can be identified by a similar scheme (14) using the reagents: iodine-potassium iodide in zinc chloride solution, alcoholic iodine, ruthenium red, and sulfuric acid. Benedict's solution can also be used to identify agar (14).

McNulty has identified agar from frozen desserts using infrared spectrometry. Agar can be distinguished from locust bean, Irish moss, pectin, gum arabic, and sodium carboxymethylcellulose by their spectra (173).

Padmoyo and Miserez have used microelectrophoresis to seperate and identify the gums, agar, arabic, tragacanth, carrageenan, carob and guar, as well as gelatin, pectin, starch, dextrin, sodium alginate, methylcellulose, and carboxymethylcellulose. The electrophoresis is carried out on cellulose acetate strips. Substances with similar motility are differentiated by staining (201).

Becker suggests a scheme for identification of agar-agar in foodstuffs using paper chromatography of the sugar after hydrolytic cleavage. Hydrolysis is made with sulfuric acid and boiled for 3 hrs. After removal of sulfuric acid by barium hydroxide and concentration of the filtrate under vacuum, the sample is chromatographed. Agar-agar is identified by the presence of galactose. Pectin, alginate, carob-seed flour, gum arabic, and tragacanth can also be identified by this method. When several thickeners are present, identification on the basis of the chromatogram can become difficult. It then seems preferable to make identification less through the spots that do show but initially through those absent (18).

VIII. Occurrence

Algae Yielding Agar and Agaroids

Name	Location
Acanthopeltis japonica	Japan
Agardhiella tenera	Vancouver Island, Canada
Ahnfeltia gigartinoides	Baja Calif., Mexico
Ahnfeltia plicata	Japan, Mexico, Russia
Ceramium boydenii	Japan
Ceramium crassum	Japan
Ceramium hypnaeoides	Japan
Ceramium rubrum	Japan
Digenea simplex	Florida
Endocladia muricata	S. Calif.
Eucheuma amakusaensis	Japan
Eucheuma crustaeforme	Japan
Eucheuma gelatinae	Japan
Eucheuma isiforme	Florida
Eucheuma muricatum	Japan
Eucheuma speciosum	Australia
Furcellaria fastigiata	Denmark
Gelidiella acerosa	Japan
Gelidium amansii	Japan
Gelidium arborescens	S. Calif.
Gelidium cartilagineum	U.S.A., Mexico, S. Africa, New Zealand
Gelidium corneum	S. Africa, Portugal, Spain, Morocco, Italy
Gelidium coulteri	Mexico
Gelidium crinale	Japan
Gelidium divaricatum	Japan
Gelidium japonicum	Japan
Gelidium latifolium	Black Sea, Eire
Gelidium liatulum	Japan
Gelidium lingulatum	Chile
Gelidium linoides	Japan
Gelidium micropterum	India
Gelidium nudifrons	Calif.
Gelidium pacificum	Japan
Gelidium planiusculum	Japan

Gelidium pristoides	S. Africa
Gelidium pulchellum	Eire
Gelidium pusillum	Japan
Gelidium serrulatum	Venezuela
Gelidium spinulosum	Morocco
Gelidium subcostatum	Japan
Gelidium subfastigiatum	Japan
Gelidium tenue	Japan
Gelidium vagum	Japan
Gigartina asperifolia	Calif.
Gigartina canaliculata	Calif.
Gigartina corymbifera	Calif.
Gigartina mamillosa	Spain
Gigartina stellata	Britain
Gracilaria blodgettii	Japan, Florida,
Gracilaria cervicornis	Florida
Gracilaria chorda	Japan
Gracilaria compressa	Japan
Gracilaria confervoides	Black Sea, S. Africa, Ecuador, N. Carolina, Trincomalee, Ceylon, Japan
Gracilaria cornea	Brazil
Gracilaria corticata	S. India
Gracilaria crassa	S. India
Gracilaria dura	Morocco
Gracilaria foliifera	S. India
Gracilaria gigas	Japan
Gracilaria henriquesiana	Ghana, Africa
Gracilaria lichenoides	Japan, Kalpitiya, Ceylon
Gracilaria multipartita	Florida
Gracilaria panamensis	Galapagos Islands
Hypnea musciformis	Florida, Brazil, S. India
Phyllophora clevelandii	Mexico
Phyllophora nervosa	Russia
Pterocladia capillacea	Japan, Egypt, New Zealand
Pterocladia densa	Japan
Pterocladia lucida	New Zealand
Pterocladia nana	Japan
Pterocladia tenuis	Japan
Sarconema filiforme	India
Spyridia	Italy
Suhria vittata	S. Africa

Biological Data

I. Acute Toxicity

None

II. Short-term Studies

Chick

Vohra and Kratzer found no effect of agar on the growth rate of day-old Arbor-Acres chicks (unspecified number) when fed ad lib at a level of 2% of the diet for a period of 20 days (279).

Rats

Groups of 6 male albino rats with an average initial live weight of 53 grams each, were individually housed and maintained for 10 weeks on a diet supplemented with 0, 5, 10, 20, or 30% agar. Food and water were provided ad lib and the intake of each was recorded. In general the rats grew very well, the only significant variation in mean daily gain in weight being found in the group fed 10% agar. These animals gained about 20% faster than the controls, possibly as a result of a beneficial effect of the agar on the functioning of the intestinal tract. It was noted that the animals fed the 20 and 30% agar diets required significantly more water per gram gain in weight than did the control group (193).

Fourteen female Long-Evans rats, 23 to 25 days old, were placed in individual cages and provided with food ad lib. Eight of the rats were given a polysaccharide-free diet supplemented with 10% agar, while the other six were maintained on a control diet. The rats were raised to maturity. Growth was slightly depressed in the agar-fed rats. It was suggested that this may be due in part to the great affinity of agar for water, resulting in a decrease in the absorption and utilization of water soluble nutrients (67).

In a feeding study by Ariyoma and Takahasi, the growth of male rats weighing 40 to 45 g and fed 23% agar for 70 days was compared to that of rats maintained on a low-carbohydrate diet. It was concluded that agar contributed no nutritive value to the diet (10).

Booth et al. fed a group of 6 weanling male rats a diet containing 15% agar for a period of 62 days. The weight gain of the agar-fed rats was nearly equal to that of the controls, however, the food efficiency was significantly lower (18%) (28).

Wierda investigated the influence of dietary agar on the intestines of rats. Wistar rats were housed in galvanized iron cages with wire

bottoms and supplied with food and water ad lib. Groups (size unspecified) of rats were maintained on diets containing 10% and 30% agar for 30 and 40 weeks, respectively, with corresponding control groups fed a balanced concentrated diet. No apparent effect was observed on the body weight of the test rats. The length of the small intestine was not significantly altered by a diet containing 10 or 30% agar, but the surface area was 15% greater in the rats fed 30% agar than in the controls. A definite increase in the length of the colon and cecum was noted in the subject rats, the surface area of the colon (excluding cecum) of the rats fed the 30% agar diet exceeded that of the controls by 69% (284).

Groups of 50 weanling albino rats (Wistar strain) were maintained for 44 weeks on control diets or diets containing 30% agar. The animals were housed in screen-bottom cages and given food and water ad lib. At the end of the feeding period there was no significant difference in body weight between the agar-fed and the control groups. However, the average weight of the large intestine was 80% greater for the rats fed agar than for those on the control diet (285).

Weanling male Sprague-Dawley rats were divided into groups of six rats each and housed in individual cages. They were fed ad lib a basal ration with or without a supplement of 25% agar. After 4 weeks, the rats were sacrificed; one each day for 6 days. The organs of the gastrointestinal tract were removed and their dry weights determined. The addition of agar to the diet produced increases in the dry weight of the stomach, the cecum, and the colon plus rectum. In one of the rats, red transverse stripes were seen in the terminal half of the colon. During the 3rd week of the feeding period, a statiscally significant reduction in weight gain was noted for the agar-fed rats. Measurement of the feces revealed that the total dry weight of the feces of the agar-fed rats was increased 13-fold over that of the controls (73).

On the basis of experiments in which groups of 6 rats were fed 10% agar or other adjuvants for 82-107 days, Schulz and Thomas concluded that agar induced the deposition of fats having lower iodine values than control animals on the basal ration (231).

In a feeding study to determine the effect of agar on the fecal excretion of lipids, Schulz and Thomas fed 6 rats (3 male and 3 female), averaging 56 g in weight, a basal diet composed of 50% solvent-processed soybean oil meal, 1.5% salt mixture, 2% yeast, 10% soybean oil, 3 drops cod liver oil per rat, and cane sugar to make 100%. Agar replaced sugar for the experimental group. The amount of feces was increased by the addition of the 10% agar, however no effect was noted on the percentage retention of lipids (232).

Hove and Herndon studied the growth of rabbits on purified diets. A comparison of a number of carbohydrates indicated that a 20% supplement of agar fed for 40 days favorably influenced growth rate (123).

Man

Agar has been used as a laxative, in daily doses from 4 to 15 g, since 1905. The effeciency of agar is not great, with the effect usually not noticeable for nearly a week (251).

III. Long-term Studies

None

IV. Special Studies

Fetotoxic

Investigations by Frohberg et al. disclosed that the intraperitoneal injection of 0.2 ml of a 1% agar solution daily from the 11th to the 15th day of gestation in NMRI mice produced no fetotoxic effects. Similar treatment from the 4th to the 8th day of gestation, however, resulted in a somewhat higher percentage of resorptions in the agar-injected mice than in the controls (82).

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Biochemical Aspects

I. Breakdown

No information available from sources obtained.

II. Absorption Distribution

Nilson and Schaller reported the apparent digestibility of agar to be 28% irrespective of the level at which it is fed to rats (193).

In a study by Booth et al., in which rats were maintained on a 15% agar diet for 7 days, the digestibility of agar was determined to be 21% (28).

III. Metabolism and Excretion

No information available from sources obtained.

IV. Effects on Enzymes and Other Biochemical Parameters

Rats maintained on an agar diet (82%) were found by De and Guha to excrete a much higher level of nicotinic acid than those on a sucrose diet (82%), indicating that agar is a more favorable medium for the growth of intestinal microflora (54).

Olitzki and Koch found that agar exhibits a weak pathogenicity-enhancing effect in mice when injected intraabdominally with Shigella dysenteriae. Kaolin and other absorbing substances heightened this effect by retarding elimination of the agar from the abdominal cavity. Long persistence in the abdominal cavity and ability to absorb and destroy white cells, thereby preventing phagocytosis, are the requirements for the effect of pathogenicity-enhancing substances. Therefore, it was concluded that the observed effect was not an enhancement of pathogenicity, but a depression of the mechanism of natural resistance (199).

Increasing levels of agar in the diet of rats produces a significant progressive depression in the apparent digestibility of dry matter of the remainder of the diet. At a level of 30% agar this depression reached 10% (193).

Meuser et al. reported what agar causes the release of histamine from rabbits thrombocytes. They found that agar contains a direct and inherent histamine releaser (agaropectin) and causes indirect release by activating the plasma, thereby inducing formation of a releaser (178).

Wu reported that agar is a strong inhibitor of polynucleotide kinase, an enzyme which catalyzes the transfer of orthophosphate from ATP to the 5'-hydroxyl termini of polynucleotides. Data indicates that the sulphate group of the agar is responsible for the inhibition. The inhibition can be counteracted by the addition of either polylysine or spermine (291).

Fischer observed that the addition of 25% agar to the diet of rats produced an increase in the dry weight of the stomach, the cecum, and the colon plus rectum (73).

V. Drug Interaction

The inhibition of Staphylococcus aureus by polymyxin, neomycin, kanamycin sulfate and streptomycin sulfate was found by Hanus et al. to be adversely affected by the presence of agar (109).

Iyer and Iyer studied the effects of agar on the inhibition of Micrococcus pyogenes var. aureus by various antibiotics. Inhibition by chlortetracycline, chloramphenicol and penicillin was augmented by agar. Tetracycline and oxytetracycline inhibition was not altered, while the inhibition by streptomycin was reversed (134).

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ON THE RELATIVE NUTRITIVE VALUE OF VARIOUS CARBOHYDRATES AND RELATED COMPOUNDS.

By

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(Received Dec. 11th., 1929).

The authors determined the relative nutritive value of different carbohydrates and related compounds by feeding experiments with albino rats by some different method from that of Ozaki. (Nippon Nogeikwagaku-kwai-shi, **2**, 845, 1926). For this purpose the following 29 compounds were used:

Starch, Agar-Agar, Inulin, Dextrin, Glycogen, Funkan, Konjak powder, Saccharose, Maltose, Lactose, d-Glucose, d-Fructose, d-Galactose, d-Mannose, d-Glucosamine, α-Methylglucoside, l-Xylose, l-Arabinose, Dioxyacetone, d-Mannite, Glycerin, Ethyleneglycol, d-Gluconic acid, d-Saccharic acid, Mucic acid, Glyceric acid, Ethylalcohol, d, l-Alanine, l-Phenylalanine,

The basal ration used in this experiment consisted of:

Meat protein	27%
Butter	70%
McCollum salt mixture	3%

(As the source of vitamin B, 0.2 g. of dry yeast was given per day per rat.)

Thus the diet was very poor on carbohydrate, nevertheless the albino rats fed on this diet ad. libitum attained nearly normal growth, as shown in Chart I (A). But when it is reduced to 4.2 g. daily the animals grew up to 80~90 g. but no further. (Chart I (B))

Experiment I.

The carbohydrates tested were:

Starch, Agar-Agar, Inulin, Dextrin, Saccharose, Maltose, Lactose.

Feeding period: April to July 1928.

Average room temperature: 23C.

Young male albino rats of 40 to 45 g. were kept in a wire cage separately and were previously fed with 4.2 g. of the basal ration for about one week. Then each 1.25 g. of the samples to be tested were supplimented to it.

The rate of growth of animals during 70 days was as follows: (Chart II)

Samples	Average gain of the body weight	
	for 70 days (g.)	per day (g.)
Maltose	114.0	1.63
Starch	95.7	1.37

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Dextrin	89.3	1.28
Lactose	89.0	1.27
Saccharose	84.3	1.26
Inulin	79.8	1.14
Agar-Agar	60.0	0.86
Basal ration	62.5	0.89

As the above result shows, the relative value is in the following order: Maltose, Starch, Dextrin, Lactose, Saccharose, Inulin and Agar-Agar; Maltose being the best. The addition of Agar-Agar had almost no effect upon the growth.

Experiment II.

In the second experiment the following substances were tested:

Glucose, Fructose, Galactose, Mannose, α-Methylglucoside, Xylose, Arabinose, Mannite, Glycerin, Ethyleneglycol. (For the control group, Starch and Dextrin were simultaneously tested.)

Feeding period: May to August 1928.

Average room temperature: 27°C.

In this experiment the animals were fed with 4.2 g. of the basal ration, as long as the animals continue to grow and when the growth was stopped 1.25 g. of each sample were added to the basal ration, then the animals began to grow up again.

Thus, the gain of the body weight after the addition of the samples may be taken as the measure of their nutritive value. The average body weight attained with 4.2 g. of the basal ration was 95 g. and the period needed for it was 41 days.

The average gain of the body weight for 40 days after adding the samples was as follows: (Chart III.)

Samples	Average gain of the body weight. for 40 days (g.)	per day (g.)
Fructose	55.0	1.38
Glucose	52.5	1.31
Starch	52.0	1.30
Dextrin	51.3	1.28
Glycerin	49.0	1.23
Arabinose	35.7	0.89
Xylose	35.2	0.88
Galactose	34.2	0.86
Mannose	32.8	0.82
Mannite	25.8	0.65
α-Methylglucoside	19.5	0.49

As is shown in the above table, the relative value of the samples tested is in the following order: Fructose, Glucose, Starch, Dextrin, Glycerin, Arabinose, Xylose, Galactose, Mannose, Mannite and α-Methylglucoside, the last named being the lowest. Ethyleneglycol was found to be toxic. All the animals fed with 1.25 g. of this substance died on the next day. Contra-

ry to the statements of some observers, Arabinose and Xylose are obviously nutritive and neither diuretic-nor diarrhetic action were observed when the amount of 1.25 g. of them were administered daily to rats. Mannose is also said to be diarrhetic, but such cases were not observed.

Experiment III.

The substances tested were as follows:

Gluconic acid, Saccharic acid, Mucic acid, Glyceric acid, Glycogen, Funkan, Konjak, Glucosamine, Dioxyacetone, Alanine, Phenylalanine, Ethylalcohol. For the control group, Starch and Glucose were simultaneously tested. (Gluconic-, Saccharic-and Glyceric acid were given as Ca-salts, Mucic acid as Na, K-salt and Glucosamine as HCl-salt.)

Feeding period: January to April 1929.
Average room temperature: 16.5°C.

The experiment was carried out exactly in the same way as in the second experiment. The maximum body weight of the animals fed on 4.2 g. of the basal ration was in average 80 g.

The average gain of the body weight for 40 days after adding the samples was as follows: (Chart IV.)

Samples	Average gain of body weight.	
	for 40 days (g.)	per day (g.)
Ethylalcohol	49.0	1.23
Funkan	[illegible].7	0.97
Glucose	35.3	0.88
Starch	34.3	0.86
Glycogen	32.7	0.82
Alanine	21.8	0.55
Konjak	19.5	0.49
Gluconic acid	19.5	0.49
Saccharic acid	19.3	0.48
Dioxyacetone	7.2	0.18

As is shewn in the above table, Ethylalcohol secured the best growth, followed by Funkan and Glycogen as well as Glucose and Starch. Alanine, Konjak, Gluconic acid and Saccharic acid are far inferior to Glucose, Dioxyacetone being least effective. Mucic acid, Glyceric acid, Glucosamine and Phenylalanine are found to be noxious. The animals fed with 1.25 g. of them have all died after about 2 to 25 days.

When the rats were fed with 1.25 g. of absolute Alcohol mixed with basal ration, they ate it up within 30 minutes and showed no signs of drunkness moreover, the growth was far better than on Glucose and Starch.

From these results, the albino rat seems to have relatively high resistance toward Ethylalcohol and it can assimilate fair amount of alcohol.

Assimilability of the carbohydrates tested:

At the end of the feeding period the urine of the animals excreted dur-

ing 18 hours after the ingestion of the carbohydrates was collected and it was tested qualitatively for reducing substances with Benedict's reagent.

The result of the test was as follows:

Carbohydrates	Reducing power of the urine	Carbohydrates	Reducing power of the urine
Starch	—	Glucose	—
Dextrin	—	Fructose	—
Inulin	—	Galactose	+++
Agar-Agar	—	Mannose	+++
Glycogen	—	α-Methylglucoside	+
Konjak	—	Glucosamine	+++
Saccharose	—	Arabinose	+++
Maltose	—	Xylose	+++
Lactose	+++	Dioxyacetone	+++

The urine of the animals fed with Lactose, Galactose, Mannose, α-Methylglucoside, Glucosamine, Arabinose, Xylose and Dioxyacetone reduced the reagent. Thus the above mentioned carbohydrates were excreted partially in the urine without being utilized by the animals and the low assimilability of these carbohydrates may be regarded as a caure of their relatively poor effect upon the nutritive value. In spite of its low assimilability, Lactose was found much effective on the growth of the rats especially in the early part of the feeding period.

Summary.

(1) The basal ration used in the feeding experiments consisted of 27% Meat protein, 70% Butter and 3% McCollum salt mixture. (As the source of vitamin B, 0.2 g. of dry yeast was given per day per rat.) Male albino rats were fed with 4.2 g. of the basal ration and 1.25 g. of different kinds of carbohydrates and other substances to be tested. Observing the rate of growth of the animals, the nutritive value of the samples were compared.

(2) The order of the nutritive value of the substances tested is as follows: As to Polysaccharide and Disaccharide, Maltose is most effective, followed by Starch, Dextrin, Lactose, Saccharose, Inulin, Glycogen and Funkan, and Konjak is far inferior to them and Agar-Agar is entirely invalid.

About Monosaccharides, Fructose and Glucose are most effective as well as Starch and Galactose, Mannose, Arabinose and Xylose come next, and Dioxyacetone and α-Methylglucoside are least effective; Glucosamine is noxious at the amount of 1.25 g. As to the effectiveness of Alcohols, Ethylalcohol seems to be most nutritive one and Glycerin follows it ranking with Starch, while Mannite is far inferior to the latter. Ethyleneglycol is poisonous, when fed with the amount of 1.25 g. of it.

As to the Acids, the nutritive value of Gluconic acid, Saccharic acid and

Alanine is half of Glucose and Mucic acid, Glyceric acid and Phenylalanine are noxious at the amount of 1.25 g.

(3) Pentoses i. e., Arabinose and Xylose are found to be nutrive under our experimental condition.

(4) At the end of the feeding period, the urine of the animals was tested for reducing substances with Benedict's reagent and with the urine of Lactose, Galactose, Mannose, α-Methylglucoside, Glucosamine, Arabinose, Xylose and Dioxyacetone positive results were obtained.

The relatively low assimilability of these carbohydrates may be regarded as a cause of their low nutritive value. In spite of its low assimilability, Lactose was much effective on the growth of the animals, especially in the early part of the feeding period.

Chart I.

The increase of body weight of rats on basal ration.

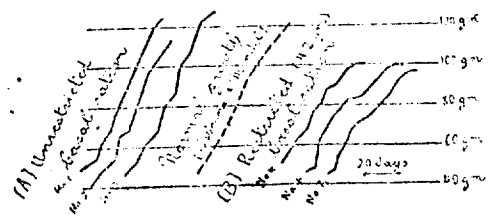


Chart II.

Graphic records of the increase of body weight of rats after adding samples (Experiment I.).

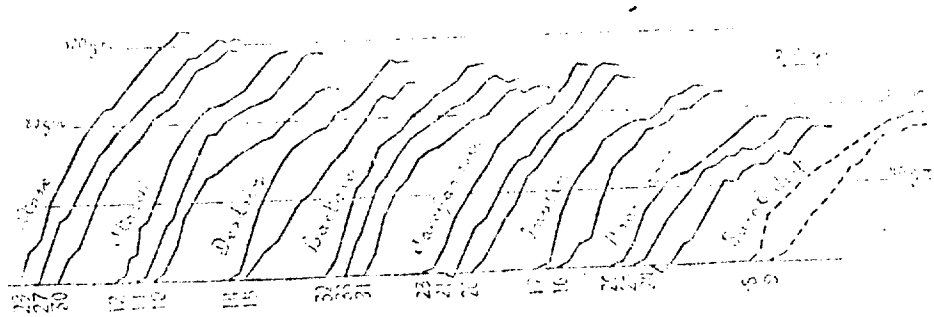


Chart III.

Graphic records of the increase of body weight of rats after adding samples. (Experiment II.).

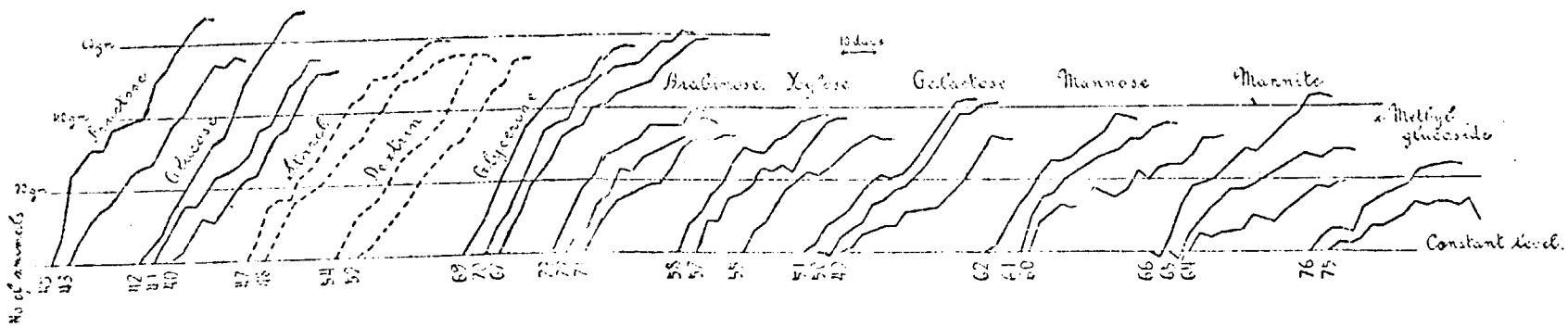
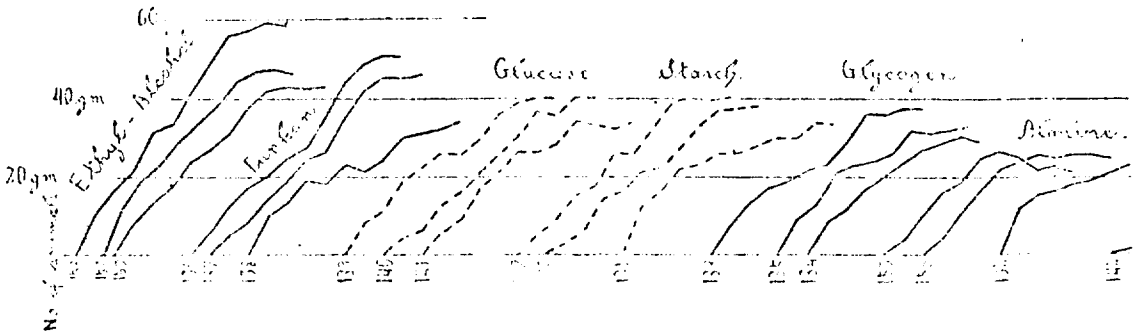
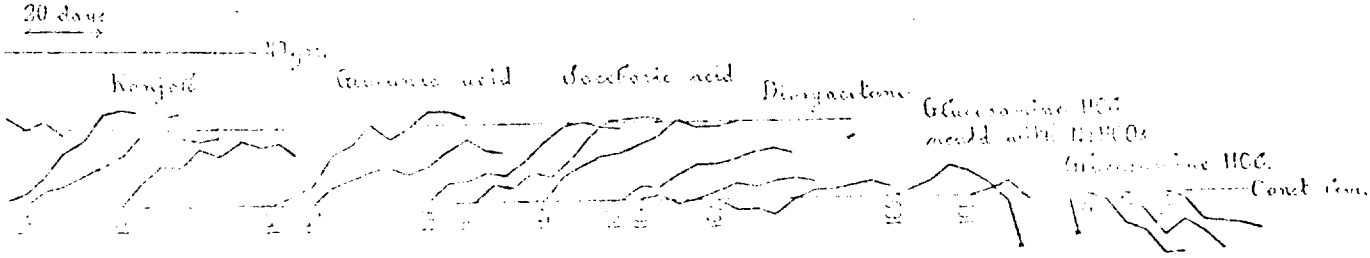


Chart IV.

Graphic records of the increase of body weight of rats after adding samples. (Experiment III.).





Source: Z. Lebensm.-Untersuch. & Forsch. 104, 187-192 (1956)
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IDENTIFICATION BY PAPER CHROMATOGRAPHY OF SWELLING AGENTS IN FOODSTUFFS

Testing for swelling agents or thickeners in pure form presents no difficulties. A compilation by Letzig (1) lists a number of precipitating reactions which permit distinguishing between the several thickeners in simple manner. However, these compounds seldom exist in such pure form that the use of such precipitating reactions, presented in synoptic form, permits non-ambiguous conclusions. Even the occurrence of two different swelling agents simultaneously can make a clear identification difficult; when there are more than two, this is true even more so. However, not only a mixture of these substances complicates the tests but also the presence of other compounds among which the proteins are predominant. Letzig's reagents are mainly protein precipitants so that precipitation will always occur in the presence of proteins. It is easy, of course, to determine the presence of proteins but this does not yet allow us to decide whether, in the absence of proteins, precipitation will have been due to thickeners. Prior to testing, proteins must therefore be removed and Wyler (2) has already described a possibility for this, specifically for meat products. This suggested removal of proteins with the aid of Carrez precipitation is so thorough, however, that not only are many swelling agents precipitated for which we must thus test prior to precipitation but the possibility of identifying those still remaining in solution is also restricted to a very few. A method of complete removal of proteins without interfering with subsequent testing and identification of swelling agents is not yet known so that the Letzig reagents in practice unfortunately have only a limited importance. As seductive as such tests are in their simplicity, they alone very rarely furnish non-ambiguous

indications of the kind of the swelling agents present. There is a possibility of precipitating the proteins with alcohol which can subsequently be easily evaporated (e.g. in vacuum) but this has the disadvantage that the proteins are not always completely eliminated and that some thickeners, specifically carob-seed flour, are also precipitated.

These disadvantages raise the question of different testing methods and Letzig already indicates in the report cited that the application of paper chromatography promises success. Since most thickeners are constituted by carbohydrates, no difficulties exist from the point of view of paper chromatography; it is merely necessary to convert the test substance into a form suitable for paper-chromatographic testing. It may happen, however, that with several swelling agents present, a non-ambiguous conclusion on the kind of the individual substances is not possible since a number of thickeners have a similar composition and/or furnish similar cleavage products after hydrolysis. In that case, however, a combination with the precipitating reagents of Letzig would permit us to continue further since the decision will lie between the few still possible thickeners. Whereas the proteins do not interfere with such paper-chromatographic tests, we must first test for the presence of carbohydrates not originating from swelling agents. Admixtures of simple sugars easily determinted with the aid of paper chromatography, can generally be removed by extracation with 50-% alcohol which does not affect the swelling agents. It is sufficient to let the substance stand with the alcohol for several hours under occasional shaking. The extract and/or the filtrate can serve to test for sugar (3) whereas the thickeners can be determined from the residue.

Hydrolysis of Swelling Agents

Since thickeners do not directly respond to paper chromatography, it is necessary to convert them to a form which is accessible to this method of testing. Among the possibilities of hydrolytic cleavage, that using enzymes is excluded and,

since carbohydrates are involved, acid hydrolysis is indicated primarily in order to prevent decomposition of cleavage products. The following conditions have been shown to be suitable and have been steadily employed for some time:

10 g of pure swelling agent and/or test substance are diluted with 50 ml of water and 50 ml of a 10-% sulphuric acid solution. If the mixture is too thick so that the retort may burst under direct heating, it should be placed in a hot water bath for a short time. When fluid, the solution is boiled in a reflux condenser where duration is a function of the kind of the probable thickeners since each substance is hydrolyzed at different rates of speed. If no indication of the type of swelling agent exists, the solution is kept at boiling temperature for three hours. It can then be directly chromatographed. However, it is preferable to concentrate if there is only a small amount of test substance; sulphuric acid also interferes somewhat, specifically with uronic acids, so that it is preferable to remove the former. For this purpose, 30 g barium hydroxide (with 8 mol water) are dissolved in 100 ml of water under heating and dripped, under constant shaking, through a filter into the hot hydrolytic solution until the latter reaches a p_H of 7. With these generally known conditions for removal of the sulphuric acid, it is easy to adjust the p_H-value as above by spotting Merck indicator paper. The precipitated barium sulphate can be rapidly filtered out, after settling, through a standard filter. Since only small amounts are required, it is sufficient if a part of the filtrate is processed further. This part is concentrated under vacuum to 50% and thus reaches again the starting concentration. If only small amounts of swelling agent are available, these can be further concentrated as desired. This solution is now suitable for paper chromatography. It is not at all necessary to keep within the quantitative relations indicated above and they can all be reduced correspondingly which is of value when only small amounts of test substance are available.

Paper-Chromatographic Conditions

Paper chromatography always takes place under the conditions already described earlier (3 & 4). The fluxing agent is n-butanol/pyridine/water (3:2:1.5) and staining is done with phtaleic acid/aniline and/or naphthoresorcinol/trichloroacetic acid. The last-named staining for non-reducing sugars plays a subordinate role in this connection. The chromatograms thus obtained with the ascending method are shown in the figure at the end of the article. The parallel obtained test solutions permit non-ambiguous identification so that even closely adjacent spots cannot be confused with each other which may easily occur when working exclusively with R_f-values.

Evaluation

Fig. 1 shows two chromatograms of some frequently occurring swelling agents. It will be clearly seen that, after hydrolysis of carob-seed flour, only the spots of galactose and mannose appear. Although mannose is nearly equal in R_f-value to mannose, it can be distinguished from the reddish arabinose by its brown color tone but these colors cannot be recognized in a black-and-white photo. With agar-agar, only galactose is clearly seen whereas xylose is manifested only as a weak spot. Gum arabic shows galactose in addition to arabinose and rhamnose. Although rhamnose is recognized only vaguely, it is characteristic since it is only infrequently found in thickeners. In the sample examined by us, there occurs further a spot immediately below the starting point which we were never able to identify. Pectin shows mainly the spot of uronic acid which can be recognized from the typical light-brown color after staining with phthalate/aniline as well as by staining with naphthoresorcinol/trichloroacetate when it turns slightly blue after some time (cf. 3 for details). We know that this involves galacturonic acid which paper chromatography cannot demonstrate since sufficient separation from glucuronic acid does not take place. The R_f-values of the two uronic acids do differ slightly and we might therefore assume that differentiation would be possible after sufficient time. Here we must take into account that the R_f-values of the two

acids are to a considerable extent a function of the concentration of these acids and of the kind of an eventually existing cation. Something similar has already been shown for some other organic acids (5). The variations so caused are greater, however, than the difference between the two R_f-values. In addition to the spot of galacturonic acid, a weak spot of glucose also occurs with pectin. It must be remembered further that a duration of hydrolysis of three hours must be strictly kept, in contrast to most other swelling agents. Whereas a duration of hydrolysis of one to two hours generally produces complete cleavage in the latter, galacturonic acid in pectin shows only weakly after one hour but increases with continued hydrolysis. Tragacanth gum shows the spots of galactose, arabinose and xylose. Comparison with the test solutions which were run parallel on the left chromatogram, makes possible rapid identification of the individual spots. The first test solution on the left shows the upper spot as galactose and the lower spot as mannose; the second test solution indicates the upper spot as arabinose and the lower as rhamnose. We already mentioned that arabinose and mannose cannot be differentiated in the figure since it is not possible to see the differences in color. The chromatogram makes it impossible to mix up the red color of arabinose with the brown color of mannose. The last test solution shows galacturonic acid at the top and, below this, xylose.

The chromatogram on the right of the figure shows "tylose" (methyl cellulose) on the left side which shows as expected the spot of glucose and below this non-hydrolyzed methylated glucoses. The occurrence of these methylated glucoses was in several preparations and appears to be characteristic for most of the tylose preparations. It is possible, however, that this is a function of manufacture and that these spots may therefore be absent some times. The adjacent cellulose glycolate also shows the spot of glucose and, in the upper part of the chromatogram, two and occasionally three unidentified spots which always have a characteristic semilunar form. Alginate exhibits only the spot of a uronic acid. The adjacent test solutions show in the

first case from top to bottom the spots of lactose, maltose and glucose whereas glucuronic acid is shown on the left.

When several thickeners are present, identification on the basis of the chromatogram can become difficult. It then seems preferable to make identification less through the spots that do show but initially through those absent. The absence of uronic acid permits a definite conclusion that neither pectin nor alginate exist. However, we believe sufficient concentration of the hydrolyzed solution must be made in order to avoid that a uronic acid does not show by reason of excessive dilution. The absence of galactose excludes the presence of carob-seed flour, agar-agar, gum arabic and tragacanth. Absence of glucose means that neither tylose nor cellulose glycolate, generally also no pectin and obviously no swelling starch can be present. Lack of mannose would exclude carob-seed flour and that of arabinose would eliminate gum arabic and tragacanth. Here we must take into account, however, that the presence of one of the two last-named spots does not permit a conclusion on the absence of the other since a possible superposition may exist which does not allow this.

With this method of selection, most of the known swelling agents can easily be excluded so that we do obtain in general a non-ambiguous picture of the composition of the mixture of swelling agents. Where in complicated cases several interpretations are possible, we can turn to the precipitating reactions Letzig mentioned above for clarification of any remaining doubts, in spite of the interferences indicated. When direct interpretation is not possible, the method of selection has proved itself also for the Letzig reactions. In most cases, however, paper chromatography should be sufficient or at least give decisive indications. As in most investigative methods, a certain intuitive judgment will develop with practice.

As described, such testing for swelling agents is obviously restricted only to those whose composition includes carbohydrates. Although these represent the greater number, this does not allow us to neglect others, e.g., gelatine. Testing for such substances

by specific paper chromatography would evidently have to be carried out from other viewpoints.

Conclusion

1. The precipitating reactions in testing for swelling agents are excellent for pure compounds but rarely furnish non-ambiguous findings for mixtures and in the presence of other substances, e.g. proteins.

2. Since most of the swelling agents are constituted by carbohydrates, paper chromatography of the sugars and/or sucroid compounds offers the possibility of identifying swelling agents by hydrolytic cleavage.

3. Hydrolysis is made with sulphuric acid and boiling at an average duration of 3 hours. After removal of sulphuric acid by barium hydroxide and concentration of the clear filtrate under vacuum, the test is made with a method described in an earlier communication.

4. Evaluation is best made by a method of selection in which all swelling agents are excluded which cannot exist due to the absence of a spot. However, it is necessary to take into account the ratios of concentration.

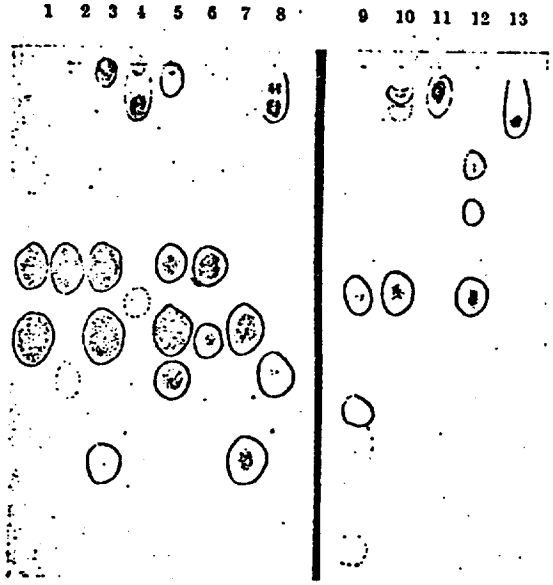

Abb. 1. *Mit Schwefelsäure hydrolysierte Quellstoffe und entsprechende Testlösungen.* Fließmittel: n-Butanol/Pyridin/Wasser (3:2:1,5), Färbung: Phthalsäure/Anilin. *Linkes Chromatogramm:* (von links) *1* Johannisbrotkernmehl, *2* Agar-Agar, *3* Gummi-arabicum, *4* Pektin, *5* Traganth. Testlösungen: *6* Galaktose + Mannose, *7* Arabinose + Rhamnose, *8* Galakturonsäure + Xylose. *Rechtes Chromatogramm:* (von links) *9* Tylose, *10* Celluloseglykolat, *11* Alginat. Testlösungen: *12* Lactose + Maltose + Glucose, *13* Glucuronsäure

Fig. 1 - Chromatogram of swelling agents hydrolyzed with sulphuric acid and the resulting test solutions. Fluxing agent: n-butanol/pyridine/water (3:2:1.5). Staining: Phthaleic acid/aniline. Left side: 1= carob-seed flour; 2 = agar-agar; 3 = gum arabic;

4 = pectin; 5 = tragacanth; test solutions: 6 = galactose + mannose; 7 = arabinose + rhamnose; 8 = galacturonic acid + xylose. Right side: 9 = tylose; 10 = cellulose glycolate; 11 = alginate; test solutions: 12 = lactose + maltose + glucose; 13 = glucuronic acid.

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TOXICOLOGY AND APPLIED PHARMACOLOGY 5, 478–484 (1963)

Physiologic Effects of Three Microbial Polysaccharides on Rats

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Received November 1, 1962

Recently two polysaccharides of unusual constitution and properties (Jeanes *et al.*, 1961b) have been produced under practical conditions (Anderson *et al.*, 1960; Rogovin *et al.*, 1961a,b) by microbial action on glucose. These biosynthetic, high molecular weight substances are water soluble and give stable, viscous solutions (Jeanes *et al.*, 1961a,b). Possible applications include their use as thickening and stabilizing agents for food and pharmaceutical products. Dextran, another high molecular weight polysaccharide, is produced biosynthetically from sucrose (Sohns *et al.*, 1954). The water-soluble dextran produces solutions of relatively low viscosity (Jeanes *et al.*, 1948). It has been shown to be a source of liver glycogen in rats (Bloom *et al.*, 1952). A partially degraded dextran for intravenous infusion has been the subject of metabolic study (Terry *et al.*, 1954).

Presented in this report are acute toxicity studies with mice and subacute toxicity studies with rats, of the recently produced polysaccharides. They include paired feeding tests and digestibility and caloric availability studies. For comparative purposes, dextran, gum guar, pectin, gum arabic, and agar were also included.

MATERIALS AND METHODS

Materials

Three exocellularly produced microbial polysaccharides were supplied by the Northern Regional Research Laboratory, Peoria, Illinois, as follows: phosphomannan Y-2448 (designated PM in this report) from the yeast *Hansenula holstii* NRRL Y-2448 (Anderson *et al.*, 1960; Rogovin *et al.*,

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1961a); polysaccharide B-1459 (designated PS) from the bacterium *Xanthomonas campestris* NRRL-1459 (Rogovin *et al.*, 1961b); and dextran B-512F from *Leuconostoc mesenteroides* NRRL B-512F (Sohns *et al.*, 1954). These products were isolated and purified in the form of dry powders of high purity and were free of viable cells. PM, the potassium salt of a diesterorthophosphomannan, is comprised of D-mannose units, orthophosphate, and potassium in the molar ratio of 5:1:1 (Jeanes *et al.*, 1961a). PS is comprised of D-glucose, D-mannose, and D-glucuronic acid (as the potassium salt) in the molar ratio of 3:2:2 and contains, in addition, about 5% *O*-acetyl groups (Jeanes *et al.*, 1961b). Dextran, a polymer of D-glucose, has 95% 1,6-linkages.

Other polysaccharides including low and high molecular weight dextrans, gum guar, agar, gum arabic, and pectin were purchased from commercial sources.

Basal diet A was a commercially formulated rat diet, to which the various polysaccharides were added at the expense of a portion of the entire diet.

Basal diet B was hand-mixed and consisted of the following ingredients (in per cent): corn meal 51.5, crude casein 11.5, linseed oil meal 10.0, alfalfa meal 2.0, cod-liver oil 3.0, bone ash 1.5, sodium chloride 0.5, and cornstarch 20.0. Test materials were fed at the expense of cornstarch.

In the initial tests, the constancy of the basal diet ingredients were maintained by substituting test materials at the expense of cornstarch only (basal diet B). Since the levels of test materials used were relatively low, substitution at the expense of the entire diet (basal diet A) was not considered objectionable, and this method was used in one test.

Methods

Acute toxicity was studied in mice by stomach tube, intraperitoneal, and intravenous administration. Albino rats were used in subacute toxicity feeding tests and in digestibility and caloric availability studies. Skin irritation and skin sensitization were studied with rabbits and guinea pigs, respectively.

Weanling albino rats from our colony were separated into uniform groups and housed with wood shavings as bedding. They were allowed free access to water and the diets indicated.

Weekly records of food intake and body weight gains were maintained. Upon termination of the feeding period, all rats were autopsied, examined

grossly, and organ weights taken. Various tissues were preserved in 10% formalin, and blocked, stained, and sectioned for histopathologic examination.

The caloric availability and digestibility of the polysaccharides were evaluated according to the procedure described by Rice *et al.* (1957). However, a modification was introduced which involved the determination of weight loss relative to the unsupplemented control group when all rats receiving supplements were switched to the basal diet only for a period of 48 hours, immediately after the 7-day supplementation period.

RESULTS

Acute Toxicity Studies

Adult mice received via stomach tube a total of 4 doses in water of 8 g PM per kilogram body weight over a period of 8 hours. Only 1 g PS per kilogram body weight could be given because of its greater viscosity. No effect of the treatments was observed. Intraperitoneal injections into mice were made daily for two 5-day periods separated by 2 days. Each injection consisted of 0.5 ml of water containing either 10 mg PM or 5 mg PS. Abdominal swelling was noted during the injection period, but during the treatment period and up to 2 weeks after the final injection there were no deaths. The absence of PM or PS in the abdominal cavity at autopsy suggests that the injected material was absorbed. A dosage of 0.2 ml of 5% PM in 0.9% saline was injected into mice via tail vein without effects other than momentarily slightly irregular breathing.

Daily topical applications of a 5% aqueous solution of either PM or PS to shaved areas of rabbit skin caused localized irritation followed by skin cracking and bleeding. The application of water-soluble cornstarch solutions produced similar effects, presumably due to continuous moistening of the skin. As the moisture evaporated, the dried material was deposited on the skin surface and appeared to be the cause of the skin fissures. Rapid healing occurred when the applications were discontinued. No evidence of skin sensitization could be demonstrated when albino guinea pigs received PM by intradermal injections daily for 10 days followed by a challenging dose 2 weeks later.

Feeding Studies

When a diet containing [illegible] PM was fed to rats, cathartic effects were observed. At levels below 5% the effect was less severe. Rats fed

a diet containing 15% gum arabic exhibited similar cathartic effects, including bulky, sticky, stringy feces. A diet containing 10% pectin also produced these effects, presumably due to the excretion of the undigested hydrophilic residue. PS at a dietary level of 15% produced abnormally

TABLE 1

EFFECT OF PM, PS, AND GUM GUAR ON GROWTH AND FOOD EFFICIENCY

Dietary treatment	Mean weight gain ± SE after 91 days[a] (g)	Food efficiency (gain/food intake)
Basal diet A	217.4 ± 6.57	0.16
+ 3% phosphomannan	203.8 ± 5.51	0.15
+ 6% phosphomannan	188.0 ± 9.84	0.14
+ 3% polysaccharide D-1459	200.8 ± [illegible]	0.14
+ 6% polysaccharide D-1459	169.2 ± 1.89	0.14
+ 6% gum guar	201.6 ± 4.88	0.14

[a] Five weanling male rats per group. Mean starting weight = 58.0 g.

large fecal pellets, which were well formed in contrast to the more fluid and sticky consistency of the feces from rats receiving PM.

Reduced food intakes and decreased growth rates, as well as abnormal feces, were observed when 7.5–15% dietary levels of PM or PS were fed. At lower levels—diets containing 3% or 6% PM, PS, or gum guar—

TABLE 2

EFFECT OF DEXTRAN, AGAR, AND GUM ARABIC ON GROWTH AND FOOD EFFICIENCY

Dietary treatment	Mean weight gain ± SE after 62 days[a] (g)	Food efficiency (gain/food intake)
Basal diet B	199 ± 16.4	0.28 ± 0.006
+ 15% agar	190 ± 11.9	0.28 ± 0.006
+ 15% gum arabic	224 ± 7.8	0.28 ± 0.005
+ 15% dextran B-512b	197 ± 13.9	0.27 ± 0.005

[a] Six weanling male rats per group in individual cages. Mean starting weight = 57.5 g.

weight gains were not significantly below those of the unsupplemented group (Table 1).[2] No significant alterations in hemoglobin or red and white cell counts were observed in these rats. Likewise, no abnormalities

[2] The statistical test used was Student's *t* test.

TABLE 3

CALORIC AVAILABILITY EVALUATION OF VARIOUS CARBOHYDRATES

Diet	Amount of supplement (g per day)	Weight change[a] (g): 7 Days on supplement	Weight change[a] (g): 2 Days on basal[c]	Net[d]	Fecal weight increase (g dry wt.)	Digestibility[b] (%)
Basal (5 g)	—	— 3	— 6	— 9	—	100
+ glucose	0.25	+ 4	— 6	+ 7	0	100
+ glucose	0.5	+ 11	— 7	+ 13	0	100
+ glucose	1.0	+ 21	— 12	+ 18	0	100
+ dextran B-512F	0.1	+ 18	— 8	+ 19	3.1	78
+ polysaccharide V-2118	0.1	+ 10	— 16	+ 12	(diarrhea)	—
+ polysaccharide B-1159	0.1	+ 4	— 20	— 8	14.5	0
+ gum guar	0.1	+ 6	— 14	+ 1	3.3	76
Basal (5 g)	—	+ 15	+ 3	+ 18	—	100
+ D-glucose	0.5	+ 38	+ 1	+ 21	0	100
+ D-glucose	1.0	+ 52	— 1	+ 33	0	100
+ pectin N.F.	1.0	+ 35	— 18	— 3	28.3	19
+ D-mannose	0.75	+ 31	— 5	+ 28	2.0	92
Basal (5 g)	—	0	— 2	— 2	—	100
+ D-glucose	0.5	+ 7	— 2	+ 7	0	100
+ D-glucose	1.0	+ 27	— 1	+ 28	0.5	99
+ dextran, low mol. wt.	1.0	+ 28	+ 1	+ 31	3.7	90
+ dextran, high mol. wt.	1.0	+ 21	— 5	+ 13	4.7	86
+ gum arabic	0.25	+ 19	— 3	+ 16	7.4	71
+ dextrin	0.25	+ 8	— 5	+ 5	20.8	21

[a] Average for 5 rats each.

[b] Digestibility = (total intake of test material minus increase in fecal weight) total intake of test material.

[c] Two days on basal followed 7 days on supplement.

[d] Adjusted for weight changes in rats on basal diet throughout.

were noted when organ weights were evaluated. The tissues of rats that had ingested diets containing 15% PM or PS for 91 days were subjected to histologic examination by Dr. William E. Ribelin, veterinary pathologist. There was no evidence of pathology.

Weight gains and food efficiencies were determined for rats ingesting high levels (15%) of agar, gum arabic, and dextran (Table 2). While the food efficiency of the group on agar was significantly lower, the weight gain was nearly normal.

A paired-feeding experiment compared the growth of rats ingesting ad libitum a diet containing 7.5% PS with comparable rats restricted to the same intake of the basal diet reduced by 7.5%. At the end of 18 days the weight gains were identical for the restricted and ad libitum groups, thus indicating the absence of any growth-inhibiting factor.

The weight gains of rats receiving polysaccharides while on a restricted caloric intake are shown in Table 3. For comparison, a group receiving no supplement and groups receiving measured amounts of a standard caloric source, such as glucose were included in each trial. D-Mannose was included in these studies because it is a major component of both PM and PS.

The weight gains shown in column 3 of Table 3 suggest that all the polysaccharides are more utilized as energy sources. However in earlier experiments the weight gains on supplemented groups were sometimes higher than was theoretically possible. This was believed to be due to the weight of hydrated undigested residues in the gastrointestinal tract. To check this possible response, the weight losses of rats that were returned to the basal diet, net losses or no gain were observed for the PS, gum guar, and pectin-supplemented groups (column 5). These materials, apparently, are not utilized. This conclusion is substantiated in the case of PS and pectin by the finding that practically all the PS and pectin fed during the 7-day period could be accounted for in the feces (column 6). Only in the case of gum guar is there a contradiction, in that digestibility was good (76%), yet utilization based on weight gain was very low (= 1).

SUMMARY

Catharsis and reduced rates of growth were the only detectable effects produced in rats ingesting diets containing 15% phosphomannan V-118 or polysaccharide B-1159 during a 91 day period. A third microbial polysaccharide, dextran B-512F

did not produce the same effects. Digestibility and caloric availability assays indicate that dextran was highly digestible and utilized as a source of energy for growth, whereas polysaccharide B-1459 was nondigestible, being completely accounted for in the feces. Phosphomannan Y-2448 was partially utilized.

Data on gum guar, pectin, gum arabic, and agar are included for comparative purposes.

Acute toxicity tests of phosphomannan and polysaccharide B-1459 and skin irritation and sensitization tests were negative.

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STUDIES ON THE BIOSYNTHESIS OF B-VITAMINS.

Part IV.

EFFECT OF DIFFERENT CARBOHYDRATES AND SOME INORGANIC AND ORGANIC NITROGENOUS COMPOUNDS ON THE BIOSYNTHESIS OF NICOTINIC ACID IN ALBINO RATS.

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[Received for publication, February 12, 1951.]

INTRODUCTION.

In a previous communication by De and Datta (1951) has been reported the influence of different nitrogenous precursors on the microbial synthesis of nicotinic acid in the rat intestine. The present investigation aims to study the effect of the dietary carbohydrate component on the biosynthetic process.

Very little work is reported in this line. Krehl *et al.* (1946, 1946*a*, 1946*b*) by growth studies on rats have observed marked superiority of dextrin, glucose and starch over sucrose in protecting against the deleterious effect by corn and they had attributed this effect to their influence in decreasing the tryptophane requirement. Schweigert and Pearson (1948) found that the diet with cerelose as the sole carbohydrate and with casein from 12 to 18 per cent level produced more growth rate, more nicotinic acid and N^1-methyl-nicotinamide excretions in rats than the other diet prepared with sucrose. This beneficial effect of cerelose was suggested to be due to its capacity for producing favourable medium for the microbial synthesis

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of nicotinic acid or its precursor in the intestine. Since in almost all the above-mentioned experiments only the growth rates as influenced by the carbohydrates have been studied and, moreover, as some protein components which possibly supplied some nicotinic-acid precursors were also included in the diets of the rats, the actual effect of the carbohydrate on the intestinal synthesis of this growth factor by microflora cannot, therefore, be known from their results.

In the present investigation, diets free from nitrogen and nicotinic acid have been prepared with different carbohydrate sources, such as glucose, sucrose, starch, agar-agar and dextrin, and their effects on the biosynthesis of nicotinic acid in rats studied.

The present work deals with the study of the effect of some inorganic nitrogenous compounds on the biosynthesis of nicotinic acid. Huff and Perlzweig (1942) in their attempts in this line have shown that ammonium lactate increased the nicotinic-acid output only when given intraperitoneally but not when given orally, thus indicating the incapability of the intestinal microflora to synthetize nicotinic acid from this compound. On the contrary, Ellinger and Abdul Kader (1949) have shown that *B. coli* isolated from rat fæces can easily produce nicotinamide when incubated with the above ammonium salt. The present investigation is an attempt to get additional information in this line and, therefore, two inorganic nitrogenous compounds, i.e. potassium nitrate and ammonium sulphate, have been chosen for this part of the work.

The present communication also reports as to whether the laboratory method of synthetizing nicotinamide, as devised by Bovarnick (1943, 1944) by heating aspargine with the amino acids, such as glutamic acid, methionine, ornithine, proline, etc., can be duplicated in the animal body when the above compounds are fed along with the basal diet.

Experimental.

Experiment with different carbohydrate diets.—The experiments of this series were carried out with 15 adult rats weighing between 180 g. and 230 g. After a fasting period of two days, they were first given the starch diet D-I and after a preliminary period of four days on this diet their urine and fæces were collected for three consecutive days. The rats were then allowed a rest on the laboratory stock diet for seven days and after this, experiment was started with the dextrose diet D-II as before. The procedure was repeated with the sucrose, dextrin and agar-agar.

Experiment with nitrate and ammonium ions, asparagine and methionine.—Three groups of adult rats, each consisting of six, were selected. One group (group A) was first given the basal diet of the composition shown in Table III and the basal excretion was collected as in the previous experiment. After the basal period, the rats were given a daily supplement of 500 mg. potassium nitrate and excretions on this supplement were measured for three days after preliminary period of four days. The rats of group B were similarly treated and after giving them a daily supplement of 500 mg. ammonium sulphate the excretions were measured for three days. The rats of group C were given supplements of 10 mg. of asparagine and 10 mg. asparagine *plus* 10 mg. methionine, the excretions were collected as before.

TABLE II.

Showing the effect of different carbohydrate sources on the biosynthesis of nicotinic acid.

(The figures indicate the average values of the total urinary and fæcal excretions of nicotinic acid in μg. per rat daily.)

Nature of the diet.	Total nicotinic-acid output in μg.
Starch D–I	72·9 ± 1·892
Dextrose D–II	75·3 ± 2·021
Sucrose D–III	62·7 ± 1·763
Dextrin D–IV	73·4 ± 1·938
Agar-agar D–V	78·0 ± 1·658

TABLE III.

Showing the effect of potassium nitrate, ammonium sulphate, asparagine and asparagine plus *methionine on the biosynthesis of nicotinic acid.*

(The figures represent the average values of the total urinary and fæcal nicotinic acid in μg. per rat daily.)

Group of the rats.	Diet.	Supplement.	Period. *	Total nicotinic acid in μg.
Group A ...	Basal †	*Nil*	P–II	58·8 ± 1·236
	Basal †	500 mg. potassium nitrate.	P–IV	63·2 ± 1·452
Group B ...	Basal †	*Nil*	P–II	61·6 ± 1·098
	Basal †	500 mg. ammonium sulphate.	P–IV	79·2 ± 1·684
Group C ...	Basal †	*Nil*	P–II	64·5 ± 1·376
	Basal †	10 mg. asparagine.	P–IV	60·8 ± 1·421
	Basal †	10 mg. asparagine *plus* 10 mg. methionine.	P–VI	67·2 ± 1·624

* P–II, P–IV and P–VI indicate the experimental periods. Those which are not indicated should be regarded as the preliminary periods for the succeeding ones.

† The basal diet was composed of 73 g. corn starch, 9 g. sugar, 12 g. ground-nut oil, 4 g. Osborne and Mendel salt mixture and 2 g. cod-liver oil. The vitamin supplements were the same as shown in Table I.

On the basis of these results the observations of Krehl *et al.* (*loc. cit.*) that the corn diet could produce even better growth when prepared with dextrin, dextrose or starch than when prepared with sucrose may be explained as due to the capacity of the former three carbohydrates to produce more favourable medium than the latter for the growth of intestinal microflora.

Effect of potassium nitrate and ammonium sulphate.—The results presented in Table III show that the daily feeding of 500 mg. of potassium nitrate along with the basal diet did not affect nicotinic-acid excretion to any appreciable extent, the values were found to be 63·2 μg. and 58·8 μg., respectively, for diets with and without nitrate supplement. When, however, nitrogen was administered in the form of ammonium ion, such as ammonium sulphate, some stimulation of nicotinic-acid synthesis was noted, the excretion values obtained being in the region of 79·2 μg. and 61·6 μg., respectively, for diets with and without ammonium-sulphate supplement. This result supports the observations of Ellinger and Abdul Kader (*loc. cit.*) on nicotinamide synthesis in ammonium-lactate medium by *B. coli* isolated from rat fæces. This falls almost under the same category as plant nutrition in which case also it has been noted by Chibnall (1939) and Vickery *et al.* (1938, 1939, 1940) that in the synthesis of amide and amino acids, as required for the synthesis of proteins and other complex nitrogenous compounds, the absorption of nitrogen in the form of ammonia is primarily necessary and even when nitrate is added it must pass by reduction through nitrite and ammonia before absorption and for these processes the soil micro-organisms take an active part. The present findings of the ineffectiveness by the nitrate ion as opposed to stimulation by the ammonium ions in promoting the biosynthesis of nicotinic acid suggests that intestinal microflora, unlike the soil microflora, cannot reduce nitrate to ammonium ions.

Effect of asparagine and asparagine plus *methionine.*—The results presented in Table III further show that asparagine when given alone or in combination with methionine does not affect the nicotinic-acid excretion to any appreciable extent and this indicates that the animals cannot perform the drastic chemical reactions as are necessary for preparing nicotinamide from the above two compounds in the laboratory as processed by Bovarnick (*loc. cit.*). Similar results have also previously been reported by De and Datta (1950) showing that the feeding of nicotine, 3-3[1] dipyridyl and quinoline do not enhance the nicotinic-acid synthesis in the body of rats although in the laboratory this growth factor can be easily prepared by oxidizing these compounds with permanganate, chromic acid and other oxidizing agents.

Summary.

1. The effect of the carbohydrate sources, such as starch, glucose, sucrose, dextrin and agar-agar, on the biosynthesis of nicotinic acid has been studied by feeding the rats nitrogen-free and nicotinic-acid-free diets based on these carbohydrate sources.

2. The nicotinic-acid excretion on starch, dextrin, agar-agar and glucose was found to be much higher than that on sucrose and the significance of these findings has been discussed.

3. The intestinal microflora can easily utilize the ammonium salt for the biosynthesis of nicotinic acid but fails to do so with nitrate.

4. Asparagine and asparagine *plus* methionine failed to stimulate the biosynthesis of nicotinic acid in the animals although in laboratory synthetic process these could be successfully utilized.

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Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

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THE STRUCTURE OF AGAR

PART I. FRACTIONATION OF A COMPLEX MIXTURE OF POLYSACCHARIDES

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(Received May 28th, 1970; accepted for publication, June 25th, 1970)

ABSTRACT

Fractionation of Difco Bacto agar on DEAE Sephadex (Cl^-) has shown that the concept that agar is made up of two polysaccharides, neutral agarose and charged agaropectin, is an over-simplification. The results indicate that agar is a complex mixture of polysaccharides all having the same backbone structure but substituted to a variable degree with charged groups. There are three extremes of structure in this spectrum of polysaccharides.

INTRODUCTION

Agar, the polysaccharide complex extracted from the agarophytes of the Rhodophyceae, is of great economic importance because of its ability to form a gel at low concentrations which can be used as a solid, usually inert, support in bacteriological media. Early chemical investigations[1,2] on the structure of agar were carried out by using the complete polysaccharide complex, and it was found to be a galactan made up of alternating (1→3) and (1→4) linkages. Araki[3] fractionated agar from *Gelidium amansii* by acetylation into two fractions, a virtually neutral polymer (agarose) and a charged fraction (agaropectin).

From the results of methylation[4] and enzymic hydrolysis[5,6], Araki deduced the structure of agarose to be repeating sequences of (1→4)-linked 3,6-anhydro-α-L-galactose and (1→3)-linked β-D-galactose residues. Agaropectin is thought to have the same repeating units[7], although some of the L-galactose residues can be replaced with sulphated galactose residues[8] and there is also partial replacement of the D-galactose residues with the pyruvic acid acetal 4,6-*O*-(1-carboxyethylidene)-D-galactose[9]. D-Glucuronic acid has also been reported to be present[10].

Araki's concept is therefore of two polysaccharides, neutral agarose and charged agaropectin. It has been generally accepted that the presence of the few charged groups always found in commercial agaroses must be due to inefficient fractionation procedures, but we now present evidence that this is not necessarily the case.

Initially, the separation of agarose and agaropectin was only a step in the structural determination of agar, but as agaropectin has been found to interfere in

several biological techniques (*e.g.*, antibiotic assays[11], immunoelectrophoreses[12], agar gel electrophoreses[13], and viral plaque formation[14]), the separation of agarose from agar has become commercially important. The acetylation procedure of Araki is expensive and difficult on a large scale and so simpler methods have been sought. Hjerten[15] precipitated the charged complex by using a quaternary ammonium salt, leaving the agarose in solution. Blethen[16] modified this process by adding carrageenan to agar in order to co-precipitate the agaropectin as the quaternary ammonium salt. Russell *et al.*[17] fractionated agar by adding polyethylene glycol (mol. wt 6000) to an agar solution to precipitate the agarose fraction. The use of DEAE cellulose in a batch process to selectively adsorb agaropectin has recently been patented by Zabin[18]. Barteling[19] found that an agarose of high purity could be prepared by washing Difco Bacto agar with EDTA and then adsorbing the agaropectin onto aluminium hydroxide. Egorov *et al.*[20] have prepared agarose by the fractionation of Difco Bacto agar with ammonium sulphate. This last process is difficult to evaluate since no analytical data are given for the product.

All these procedures were developed without a full understanding of the nature of agar. Before an optimal preparation scheme for agarose can be evaluated, more-detailed knowledge of the structure of agar is necessary.

RESULTS AND DISCUSSION

The predominant genera which give gelling, agar polysaccharides are *Gelidium*, *Gracilaria*, *Acanthopeltis*, *Ceramium*, and *Pterocladia*. Agar is extracted from an agarophyte by boiling the alga in water. On cooling, the filtered solution sets to a gel which can be purified by freezing and thawing. The thaw water contains salts, pigments, and polysaccharides. No information is available on the polysaccharide fraction removed by this initial, purification step, but it is likely that it is highly charged. The agar which has been used throughout this investigation is the commercial preparation Difco Bacto agar. This product is thought to be prepared from *Gelidium cartilagineum* by the above procedure.

Charge-dependent solubility of polysaccharides at various temperatures has been used to fractionate agar[21] and other polysaccharide complexes[22], and this technique was investigated first. Three fractions were collected and analysed. Fraction 1 was obtained by aqueous extraction at 20°, fraction 2 at 50°, and fraction 3 was the remaining polysaccharide; the analytical data are shown in Table I.

By changing the conditions of the washing procedure, products having different values for their 3,6-anhydro-L-galactose, sulphate, and pyruvate contents were obtained. Hence, it became evident that these fractions were not different polysaccharides having homogeneous structures but just fractions of a whole polysaccharide complex. The high value for 3,6-anhydro-L-galactose in Fraction 2 was found not to be due to contamination with agarose (which presumably makes up part of Fraction 3), since when Fraction 2 was added to a column of DEAE Sephadex A-50 (Cl^-), no polysaccharide could be eluted from this anion exchanger with distilled water.

TABLE I

FRACTIONATION OF DIFCO AGAR BY WASHING AT VARIOUS TEMPERATURES[a]

Polysaccharide component	*Yield* (%)	*3,6-Anhydro-L-galactose*[23] (%)	*Sulphate* (SO_4^{2-})[24] (%)	*Pyruvic acid*[25] (%)
Difco Bacto agar	100	30.0	2.96	0.91
Fraction 1, material soluble at 20°	15	5.3	7.00	0.55
Fraction 2, material soluble at 50°	25	19.4	4.10	1.30
Fraction 3, the remaining polysaccharide	50	45.0	0.95	0.21

[a]The methods of analysis are given in the appropriate references.

This suggested the second method for fractionation of agar by column chromatography using DEAE Sephadex A-50 in the chloride form. Sephadex was chosen in preference to cellulose because of its more-open structure. Difco Bacto agar was added to the column and the gel eluted with distilled water until the eluant became polysaccharide free. A gradient concentration of sodium chloride solution was then used to elute the charged polysacharides. The elution curve is shown in Fig. 1. From the analyses of selected fractions, it was shown that, with increasing ionic strength of the eluant, the sulphate content of the eluted polysaccharide increased. In a larger scale experiment, a step-wise increase in the ionic strength of the eluant

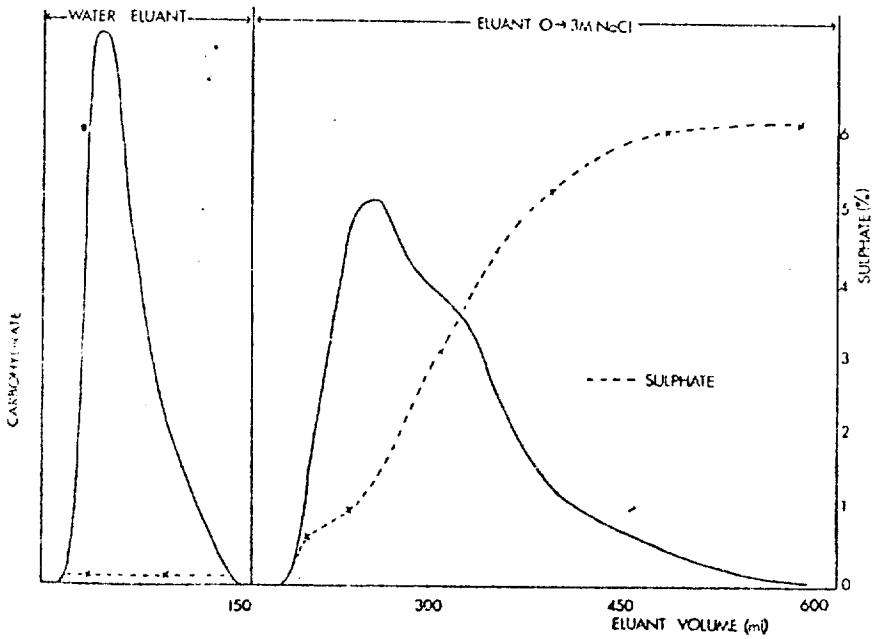

Fig. 1. Elution of Difco Bacto agar from DEAE Sephadex A-50 with a gradient concentration of sodium chloride. (———) Fractions assayed for carbohydrate with phenol–sulphuric acid; (– – – – –) fraction assayed for sulphate.

was used, and the analytical data and weights of eight fractions thus obtained are expressed in Table II.

TABLE II

FRACTIONATION OF DIFCO BACTO AGAR BY COLUMN CHROMATOGRAPHY ON DEAE SEPHADEX A-50

Polysaccharide fraction	*Eluant*	*Yield*[a] (%)	*3,6-Anhydro-L-galactose* (%)	*Sulphate* (%)	*Pyruvic acid* (%)	*Gel formed*[b]
a	distilled water	25.5	47.8	0.08	0.02	+ + + +
b	0.2M NaCl	1.9	44.1	0.94	0.06	+ + +
c	0.5M NaCl	25.5	44.0	1.07	0.09	+ +
d	0.8M NaCl	3.8	19.3	3.40	0.97	−
e	1.2M NaCl	1.9	12.0	5.80	0.64	−
f	1.8M NaCl	0.3	9.9	6.00	0.70	−
g	2.4M NaCl	0.3	9.0	6.00	0.50	−
h	3.0M NaCl	0.3	5.0	7.00	0.50	−

[a]Total yield, 59.5% of the polysaccharide added to the column; [b]+ is indicative of gel strength of a 1% solution, − no gel at a concentration of 1%.

As can be seen from the yields in Table II, a considerable proportion of material was irreversibly bound to the column. This phenomenon has been observed for other charged polysaccharide complexes[26]. The polysaccharide bound to the column possibly comprised highly charged galactans not removed in the freezing and thawing process. When this experiment was repeated with agar which had been washed at room temperature (Fractions 2 plus 3, Table I), the elution curve was the same as in Fig. 1 but the total yield from the column was 90% instead of 59.5%. When washed agar (Fraction 3) was fractionated on DEAE Sephadex (Cl^-), there was again almost total recovery with elution of components corresponding to Fractions *a*, *b*, and *c* in Table II. The total charge content of the polysaccharides eluted in Fraction *a* is least when a purified agar of low charged content is added to the Sephadex, but there is a

TABLE III

THE YIELD OF POLYSACCHARIDE VERSUS CHARGE CONTENT

Polysaccharide fraction	*Yield*[a] (%)	*Sulphate* (%)	*Pyruvic acid* (%)
Difco Bacto agar	100	2.96	0.96
Remaining polysaccharide after washing at 20° and 50° (Fraction 3)	50	0.95	0.21
Fraction 3, precipitate with polyethylene glycol (Russell *et al.*[17])	31	0.65	0.05
Polysaccharide in water eluate when Difco Bacto agar is added to DEAE Sephadex	25	0.09	0.01
Polysaccharide in water eluate when Fraction 3 is added to DEAE Sephadex	10	0.05	[b]

[a]Yield expressed as % of total agar. [b]Not detectable.

corresponding decrease in yield when expressed as a percentage of the total agar (Table III).

It was also observed that there is decreasing gel strength with increasing sulphation and decreasing content of 3,6-anhydro-L-galactose, but some of the highly charged fractions are still capable of forming a firm gel. Both these observations are in agreement with Rees' concept[27] that the gel-forming capability is due to the three equatorial hydrogen atoms on the 3,6-anhydro-L-galactose residues which constrain the polysaccharide into a helix. Interactions of helices cause gel formation. Any replacement of a sulphated galactose for a 3,6-anhydro-L-galactose residue changes the conformation of the L residue, hence causing a kink in the helix, and gelling ability is therefore impaired. From Table II, it can be seen that both the percentage of pyruvic acid and sulphate do not increase with the ionic strength of the eluant, the masking of the basic unit with 4,6-*O*-(1-carboxyethylidene)-D-galactose reaching a peak when the ionic strength of the eluant is 0.8M sodium chloride. Similarly, Fraction 2, eluted with dilute saline at 50°, has a higher pyruvic acid content than Fraction 1 which was eluted at 20° (Table I). This indicates that the masking of the basic chain with 4,6-*O*-(1-carboxyethylidene)-D-galactose groups occurs away from the sulphated galactose residues. Whether the masking occurs as 4,6-*O*-(1-carboxyethylidene)-D-galactose on some polysaccharide molecules and sulphated galactose residues on others or whether the 4,6-*O*-(1-carboxyethylidene)-D-galactose residues are laid down away from the sulphated galactose residue on one particular chain is difficult to decide.

The presence of 4,6-*O*-(1-carboxyethylidene)-D-galactose residues would not be expected to affect the gel strength as much as the sulphated units for they do not interfere with the equatorial hydrogen atoms on the 3,6-anhydro-L-galactose residues; nor do they cause kinking in a molecule as the conformation of the molecule is not altered.

The net charge on a solely "pyruvated" chain would be considerably reduced at low pH, whereas the "sulphated" molecules would still be fully ionised. An attempt was therefore made to fractionate "pyruvated" and "sulphated" molecules. The fraction eluted at 50° from agar (2, Table I) was refractionated on a column of DEAE Sephadex A-50 at pH 3.4. Because of the need to run the column at 50°, in order to prevent gelling of the polysaccharide, control of pH was necessary to avoid excessive hydrolysis of the polysaccharide. The polysaccharides were eluted with an increasing molarity of sodium chloride as shown in Fig. 2. The analytical data for the three fractions are expressed in Table IV.

A complete separation of "pyruvated molecules" was not achieved but pyruvate-enriched and sulphate-enriched fractions were obtained. Agar cannot therefore be regarded as containing two components, neutral agarose and charged agaropectin, but is a complex range of polysaccharides varying in total charge content. Agar may be regarded in terms of extremes of structure, as has recently become apparent for the carrageenan mixture of polysaccharides[28,29].

Complete agar is thought to consist of chains having alternating α-(1→3) and

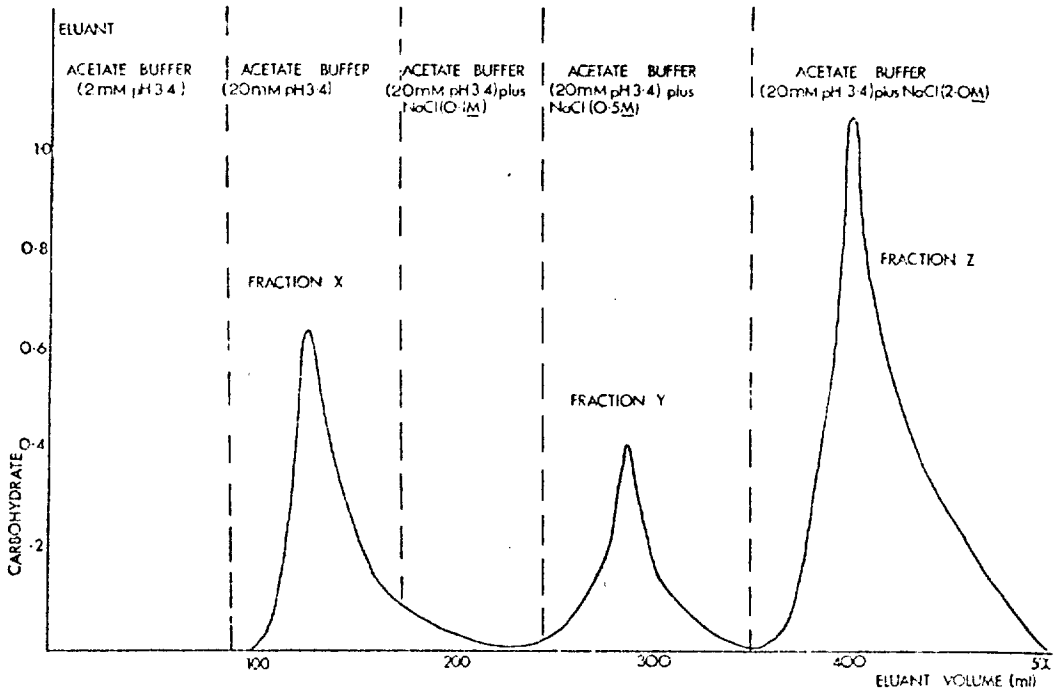

Fig. 2. Elution of Fraction 2 from DEAE Sephadex A-50 with a gradient concentration of sodium chloride in acetate buffer (pH 3.4). Assay by phenol–sulphuric acid (———).

TABLE IV

FRACTIONATION OF AGAR FRACTION 2 (TABLE I) ON DEAE SEPHADEX A-50 AT pH 3.4

Polysaccharide fraction	*Eluant*	*Yield*[a] (%)	*3,6-Anhydro-L-galactose* (%)	*Sulphate* (%)	*Pyruvic acid* (%)
Fraction 2 before fractionation	—	100	19.4	4.1	1.3
X	Acetate buffer (20mM, pH 3.4)	20	18.0	4.8	1.7
Y	Acetate buffer (20mM, pH 3.4) plus NaCl (0.5M)	15	33.3	2.0	2.5
Z	Acetate buffer (20mM, pH 3.4) plus NaCl (2.0M)	32	8.3	9.4	0.9

[a]Expressed as % of polysaccharide added to the column.

β-(1→4) linkages, and the extremes of structure are therefore as follows. (*a*) *Neutral agarose*. The idealised structure put forward by Araki of a molecule of alternating (1→4)-linked 3,6-anhydro-α-L-galactose and (1→3)-linked β-D-galactose residues containing no charge groups. The data presented in this paper suggest that this ideal molecule is present in very small proportion, if at all, in Difco Bacto agar. (*b*) *Pyruvated agarose with little sulphation*. From the first extreme of neutral agarose, the

masking of the basic structure with charged groups gradually increases. The D-galactose residues are substituted by 4,6-*O*-(1-carboxyethylidene)-D-galactose residues until the substitution reaches approximately one in twenty. At this point, the concentration of sulphate is 2%, indicative of substitution of the 3,6-anhydro-L-galactose residues with galactose sulphate. (*c*) *Sulphated galactan containing no or little 3,6-anhydro-L-galactose or 4,6-O-(1-carboxyethylidene-D-galactose residues.* From the second extreme, the substitution of the D-galactose residues by 4,6-*O*-(1-carboxyethylidene)-D-galactose residues gradually decreases and the concentration of galactose sulphate increases to the third extreme, a non-gelling, sulphated galactan.

The ratio of each extreme will be different for agars from different species and possibly with the time of year and stage in growth of the plant. The yield of each extreme can be expressed as in Fig. 3. This Figure explains why commercial preparations of agarose contain charged groups, and also explains why the yield becomes progressively less when preparing agarose with fewer and fewer sulphate and pyruvate groups (see also Table III).

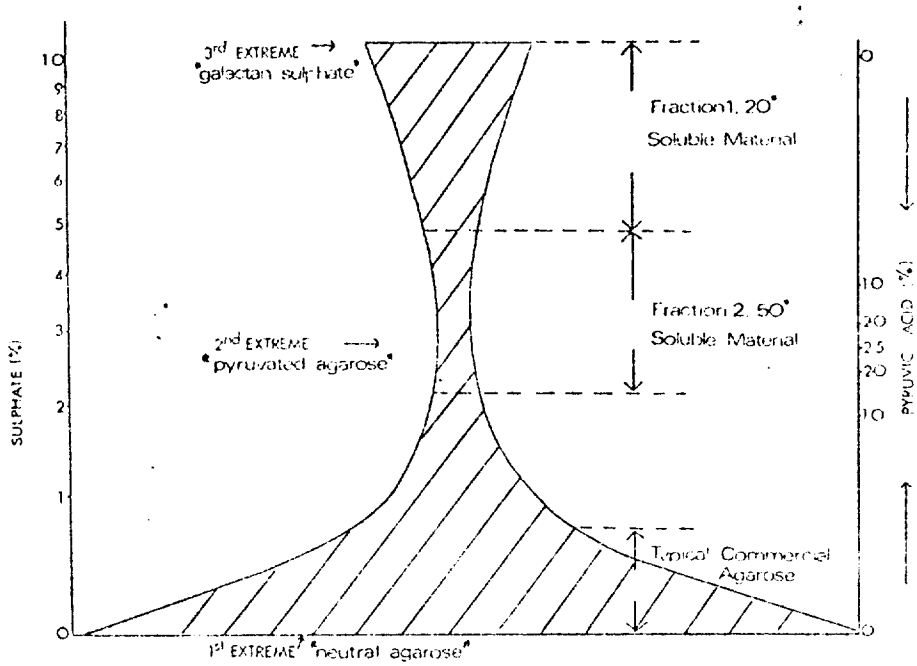

Fig. 3. The complexity of the spectrum of polysaccharides in Difco Bacto agar. The shaded area represents complete agar. The area within the curves between any lines drawn horizontally indicates the approximate amount of polysaccharide having that number of charged groups.

The practical definition of agarose will have to indicate that agarose is not a neutral polysaccharide obtained by separation from the charged polymer agaropectin. Agarose is that mixture of agar molecules having lowest charge content, and therefore the greatest gelling ability, fractionated from a whole complex of molecules, called agar, all differing in their extent of substitution with charged groups.

EXPERIMENTAL

Fractionation of agar. — (*a*) *By washing the polysaccharide at 20° and 50°.* Agar (100 g of Difco Bacto agar) was suspended in 50mM sodium chloride at room temperature in a jacketted column (50 × 8 cm). The agar was then washed with 50mM sodium chloride at 20° until the eluant became polysaccharide-free (phenol–sulphuric acid reagent[30]). The dilute solution of sodium chloride was used instead of distilled water so as to prevent excessive swelling of the polysaccharide. The polysaccharides in the eluants were precipitated with ethanol (4 volumes). Water at 50° was then circulated through the jacket, and the agar was washed with saline at 50° until the eluant was again polysaccharide-free. This fraction was also precipitated with ethanol (4 volumes). Water at 100° was then circulated through the jacket until the remaining polysaccharide was in solution. This solution was filtered to remove debris and poured into ethanol (4 volumes) to precipitate the polysaccharide. Analytical data for the fractions are given in Table I.

(*b*) *By column chromatography on DEAE Sephadex A-50.* DEAE Sephadex (Pharmacia) was prepared in the chloride form by washing with 0.5M hydrochloric acid, 0.5M sodium hydroxide, and 0.5M hydrochloric acid. The gel was then washed extensively with water to ensure that the pH of the eluant was that of distilled water. The gel was packed into a jacketted column (30 × 4 cm) maintained at 50–60°. The temperature of the eluant was also 50–60°.

A solution of Difco Bacto agar (2 g) in distilled water (90 ml) was added to the Sephadex gel. The gel was eluted with water, and fractions (10 ml) were collected until the eluant became polysaccharide-free. A gradient concentration of sodium chloride (0→3M) was then used to elute the more highly charged polysaccharide molecules. When the eluant became polysaccharide-free, the Sephadex gel was eluted with sodium chloride solution (5M) to ensure that as much polysaccharide as possible had been eluted. Appropriate fractions were combined, dialysed, precipitated in ethanol (4 volumes), and centrifuged. The polysaccharides were air-dried; the elution curve is shown in Fig. 1.

In a larger scale experiment, Difco Bacto agar (12 g) was added to a larger column of Sephadex (50 × 8 cm). The following concentrations (M) of sodium chloride were used to elute the polysaccharide fractions, 0, 0.2, 0.5, 1.8, 2.4, and 3.0 (Table II). The polysaccharides in each eluant were recovered as described above.

(*c*) *Further fractionation of Fraction* 2 (*Table I*) *on DEAE Sephadex A-50* (Cl^-) *at pH 3.4.* DEAE Sephadex (Cl^- form) was suspended in acetate buffer (2mM at pH 3.4). The Sephadex gel was then stirred for 1 h before being packed into a jacketted column (30 × 4 cm) maintained at 50°. A solution of Fraction 2 (2 g) in water (50 ml) was added to the Sephadex gel. The gel was washed with acetate buffer (2mM, pH 3.4), and the polysaccharide fractions were then eluted with acetate buffer (20mM, pH 3.4) and solutions of increasing ionic strength of sodium chloride (0.1M, 0.5M, 2.0M) in acetate buffer. The fractions were dialysed overnight against warm, running water and

then precipitated with ethanol (4 volumes). The precipitates were removed by centrifugation and air-dried before analysis (Table IV).

ACKNOWLEDGMENT

The generous support of the National Research Council of Canada is gratefully acknowledged.

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Effects of Polysaccharides on the Appetite and Efficiency of Food Utilization in the Growing Rat.

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THE PURPOSE of the present communication is to report the effects of polysaccharides on the appetite and efficiency of food utilization in the growing rat. The polysaccharides employed consisted of cellulose[2], agar agar[3] and pectin[4].

Thirty female rats of the Long-Evans strain were selected for the following experiment at 23 to 25 days of age and an average weight of 42.7 grams. Animals were placed in individual metal cages with raised screen bottoms to prevent access to feces, and feeding was continued for eight weeks. Diets listed in table 1 were fed ad lib, and records were kept of the daily food consumption of all rats.

Results are summarized in table 2. Rate of growth, gross appearance and caloric consumption on diet A was not inferior to that observed on the polysaccharide-containing rations. On the contrary, growth was somewhat depressed on the agar agar and pectin-containing rations, and at least for the latter the reduction appears significant. Depression in growth was correlated not only with reduced caloric intake but also with reduced efficiency in the utilization of ingested calories. Of the three polysaccharide-containing rations, that containing cellulose was the most satisfactory from the standpoint of growth, caloric consumption and efficiency of food utilization. It is not improbable that under the conditions of the present experiment the poorer performance of the agar agar and pectin containing rations may have been due in part to the greater affinity of these substances for water with a resulting decrease in the absorption and utilization of water soluble nutrients.

* From the Emory W. Thurston Laboratories, Los Angeles, California. Submitted June 24, 1946.

TABLE 1

COMPOSITION OF EXPERIMENTAL DIETS

	A	B	C	D
Vitamin Test Casein[1]	30.0	30.0	30.0	30.0
Salt Mixture[2]	4.5	4.5	4.5	4.5
Yeast[3]	12.0	12.0	12.0	12.0
Cottonseed Oil[4]	10.0	10.0	10.0	10.0
Sucrose	43.5	33.5	33.5	33.5
Cellulose (Cellu Flour)		10.0		
Agar Agar			10.0	
Pectin				10.0

To each kg. of the above diets were added 1.2 grams choline chloride and 5 mg. 2-methyl-naphthaquinone. Each rat also received a weekly supplement of a vitamin A-D concentrate[5] containing 100 U. S. P. units of vitamin A and 10 U. S. P. units of vitamin D.

[1]General Biochemicals, Inc., Chagrin Falls, Ohio.

[2]Salt Mixture No. 1 (Sure (1)).

[3]Brewers' Type Yeast No. 200, Anheuser-Busch, Inc., St. Louis, Mo.

[4]Wesson.

[5]Nopco Fish Oil Concentrate, assaying 800,000 U. S. P. units of vitamin A and 80,000 U. S. P. units of vitamin D per gram.

TABLE 2

EFFECTS OF POLYSACCHARIDES ON FOOD INTAKE AND EFFICIENCY OF FOOD UTILIZATION FOR GROWTH IN THE RAT

Group	Number of animals	Initial body weight (grams)	Average gain in weight for 8 week period[1] (grams)	Caloric value per gram diet[2]	Average food intake per rat for 8 week period		Average caloric intake per gram gain in weight
					Grams	Calories	
Diet A	6	41.0	137.0 ± 5.0	4.32	577.0	2496.1	18.22
Diet B	8	4[illegible].0	[illegible]	3.9	[illegible]	[illegible]	17.0
Diet C	6	4[illegible].0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Diet D	8	4[illegible].0	[illegible]	[illegible]	[illegible]	[illegible]	19.22

1. Including standard error of the mean calculated as follows: $\sqrt{\sum d^2 / n}$ where "d" is the deviation from the mean and "n" is the number of observations.

2. Computed on the basis of 4 calories per gram protein or carbohydrate and 9 calories per gram fat. It is assumed that cellulose, agar agar and pectin yield no calories to the organism.

SUMMARY

Female rats were raised to maturity on a polysaccharide-free diet and rations containing respectively 10% cellulose, 10% agar agar and 10% pectin. No beneficial effects on growth, gross appearance, appetite or efficiency of food utilization were observed on any of the diets due to the presence of the respective polysaccharides.

1. The subject matter of this paper has been undertaken in cooperation with the Quartermaster Corps Committee on Food Research.

2. Cellu Flour, Chicago Dietetic Supply House, Chicago, Ill.

3. Bacto-Agar, Difco Laboratories, Detroit, Mich.

4. N. F. Pectin, California Fruit Growers' Exchange, Ontario, Calif.

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Analytical Chemistry 24, 1952

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Identification of Stabilizing Agents

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EXTENSIVE use of a variety of polysaccharides as stabilizing or thickening agents in foods has created a need for analytical methods for the identification of commercially available materials and for their detection and quantitative estimation in food products. A number of stabilizers, which are used or have been suggested for use in foods, are listed and their chemical nature is indicated in Table I. Throughout this report the term "gums" is used in referring to all of these materials. Gelatin is included because it has many uses similar to those of the polysaccharides.

As the initial step in a project concerned with the development of the required analytical procedures, methods for the qualitative identification of a number of gums have been studied. Several procedures for the identification of gums are found in the literature. A method described by Jacobs and Jaffe (*15*) classifies the gums on the basis of physical characteristics or appearance of precipitates and thus requires considerable experience on the part of the analyst. Their outline does not include pectic substances, alginates, methylcellulose, carboxymethylcellulose, gum ghatti, starch, or gelatin. It also has the disadvantage of using an unstable reagent (Millon's) which must be freshly prepared each day. A method developed by Cannon and al

The present work was undertaken to develop a method for the identification of stabilizing and thickening agents used in food products. The materials studied were pectin, de-esterified pectin, algin, Irish moss, gum tragacanth, gum karaya, locust bean gum, starch, agar, gum arabic, gum ghatti, carboxymethylcellulose, methylcellulose, and gelatin. A proposed identification scheme is based on precipitation reactions with calcium chloride, sodium hydroxide, barium hydroxide, and lead acetate. In addition, reactions of the stabilizing agents with a cationic soap, ammonium sulfate, mercuric nitrate, papain, and gelatin are listed. The proposed scheme should be useful for identification of unknown stabilizing agents. A number of the reactions reported might be employed for the identification of individual stabilizing agents in mixtures of these materials or isolated from foods.

the Association of Official Agricultural Chemists (*3, 5*) does not provide for the identification of pectic substances, alginates, methylcellulose, carboxymethylcellulose, locust bean (carob) gum, or gum ghatti. Bryant (*4*) has described a procedure for distinguishing between pectin and certain gums, but it does not provide for positive identification of the gums. A number of other publications dealing with characteristic properties of these polysaccharides have been summarized by Mantell (*10*), but a systematic procedure is still needed for their identification. Such a procedure would be useful for the identification of products used as thickeners or stabilizers in foods, drugs, and cosmetics and ultimately for the identification of polysaccharides isolated from these materials.

In the present investigation, the manner in which the gums disperse in water after being wetted with alcohol has been a valuable index to the identity of unknown samples. Their solubility properties are summarized in Table II. Use has also been made of the fact that many of the polysaccharides occur as salts of complex organic acids (Table I). The acidic properties may be due to the presence of uronic acid groups, as in gum arabic, or to the unesterified portion of sulfuric acid molecules esterified with the polysaccharide. When mineral acids are added to aqueous solutions or dispersions of these salts, the effective concentrations of the polysaccharide anions are decreased. Thus, although the complex anions may yield insoluble salts with heavy metal cations, most are not precipitated from acid solution. The amount the pH must be raised in order to precipitate the heavy metal salts—e.g., barium, mercury, or lead—is frequently characteristic of the individual polysaccharide.

Table I. Source and Chemical Nature of Materials Commonly Used as Thickening Agents in Foods

Material	Source	Principal Components	References
Pectic substances	Fruits	Galacturonic acid (occurs as methyl ester)	(*18*)
Algin (sodium alginate)	Seaweeds	Mannuronic acid (Na salt)	(*16*)
Irish moss	Seaweeds	Galactose, galactose 4-sulfate (K and Ca salts)	(*18*)
Agar	Seaweeds	Galactose (D- and L-), galactose 6-sulfate (Ca and Mg salts)	(*18*)
Tragacanth	Plant gum	L-Fucose, D-xylose, galacturonic acid, L-arabinose, D-galactose	(*18*)
Methylcellulose	Modified cellulose	Methyl-D-glucose	(*18*)
Starch	Plants	D-Glucose	(*18*)
Carboxymethylcellulose	Modified cellulose	Carboxymethyl-D-glucose	(*18*)
Locust bean gum (carob gum)	Seed endosperm	Mannose and galactose	(*19*)
Guar gum	Seed endosperm	Mannose and galactose	(*20*)
Karaya	Plant gum	Galactose, acetic acid, galacturonic acid, rhamnose, tagatose	(*14*)
Arabic (acacia)	Plant gum	D-Glucuronic acid, D-galactose, L-arabinose, rhamnose (mixed Ca, Mg, and K salts)	(*18*)
Ghatti	Plant gum	L-Arabinose, galactose, galacturonic acid (Ca salt)	(*16*)
Gelatin	Modified proteins	Amino acids	

Table II. Dispersion in Water of Gums, Wetted with Alcohol

Gum	Manner of Dispersal in Water
Pectic acid	Insoluble
Pectate (Na, K, or NH_4 salts)	Forms either clear or turbid solution on heating
Pectate (Ca salts)	Insoluble
Pectin	Swells in cold water and dissolves on heating
Alginate	Dissolves slowly in cold water or quickly on heating to form viscous solution
Irish moss	Dissolves slowly in cold water, rapidly on heating to form viscous solution
Agar	Swells in cold water, dissolves on heating, gels on cooling
Tragacanth	Swells to form viscous dispersion in cold or hot water, but does not form true solution
Methylcellulose	Dissolves slowly in cold water but becomes cloudy or gels on heating
Starch	Disperses on heating
Carboxymethylcellulose	Dissolves slowly in cold water, rapidly on heating, giving clear viscous solution with some fibrous suspended material
Locust (carob)	Forms viscous suspension but not a true solution
Karaya	Forms viscous suspension. Insoluble parts settle on standing
Arabic (acacia)	Dissolves in cold water to form a clear or slightly viscous solution
Ghatti	Dissolves to form almost clear solution but some insoluble material may remain as fine suspension
Gelatin	Swells in cold water and dissolves on heating

EXPERIMENTAL

During the present investigation 0.5 to 1.0% aqueous dispersions of the polysaccharides were used for the tests. Aliquots of from 3 to 5 ml. were treated with varying concentrations of the reagents which it was hoped would give characteristic precipitation reactions. Initially the reagents used were those for which Jacobs and Jaffe (*15*) have described reactions with several polysaccharides. Subsequently a number of other reagents were used.

Reactions which were found useful for characterizing the gums are summarized in Tables III and IV. Only those materials having anionic components, such as alginates, or potential anionic components, such as pectin, give pronounced reactions with cationic soap (Table III). As in the case of precipitates with heavy metals, the precipitates with the cationic soap quickly disperse on acidification of the medium. Ammonium sulfate is of interest, in that it gives pronounced precipitation tests with several of the gums but not with alginates, pectin, tragacanth, karaya, arabic, or ghatti, each of which probably contains uronic acid components. The reactions with Stokes's acid mercuric nitrate illustrate the effects of low pH on precipitation of heavy metal salts of the polysaccharide acids. An excess of the reagent makes the solution strongly acidic and thus the weakly dissociated acids redisperse. Alginic acid and pectic acid are insoluble and thus are not dissolved by excess Stokes reagent. Papain and gelatin give p

Table III. Precipitation Reactions of Polysaccharide Gums and Gelatin

Gum	1 Vol. 1% Solution of Cationic Soap[a]	0.5 Vol. Saturated Ammonium Sulfate	Diluted[b] Stokes's Acid Mercuric Nitrate Added Dropwise	1 Vol. 2% Papain (6)[c]	1 Vol. 2% Gelatin[c]	1 Vol. 95% C2H5OH + 2 — 3 Drops Saturated NaCl
De-esterified pectin	Fine opaque precipitate	Gelatinous translucent precipitate	Gels (almost opaque). Insoluble in excess reagent	Precipitate	Precipitate	Gelatinous precipitate, gels (1 vol.)
Alginate	Fine opaque precipitate	Nil	Gels (almost opaque). Insoluble in excess reagent	Precipitate	Precipitate	Gelatinous precipitate (1 vol.) becomes stringy with 4 vol. alcohol
Pectin	Flocculent precipitate	Nil	Forms almost opaque gel which dissolves in excess reagent	Cloudy	No definite effect	Transparent gelatinous precipitate. Gels (1 vol.)
Irish moss	Stringy or flocculent precipitate	Gelatinous precipitate or gel	Transparent gel. Redispersed by excess reagent	Precipitate	Precipitate	Stringy precipitate
Agar	Gelatinous precipitate	Flocculent precipitate	Turbid or cloudy	Cloudy	Precipitate	Fine flocculent precipitate
Tragacanth	Fine opaque precipitate	Nil	Flocculent precipitate. Dissolves in excess reagent	Precipitate	Precipitate	Voluminous precipitate, jellylike
Methylcellulose	Nil	Precipitate	Nil	Nil	Nil	Nil
Starch	Nil	Precipitate	Nil	Nil	Nil	Opaque flocculent precipitate
Carboxymethylcellulose	Gelatinous clotted precipitate	Gelatinous precipitate	Precipitate dissolves in excess reagent	Precipitate	Precipitate	Voluminous clotted precipitate
Locust	Nil	Precipitate (voluminous)	No pronounced effect	No pronounced effect	Nil	Voluminous opaque stringy precipitate, forms clot
Karaya	Flocculent precipitate	Nil	Flocculent precipitate dissolves in excess reagent	Precipitate	Precipitate	Flocculent precipitate, discrete particles
Arabic (acacia)	Precipitate (very fine)	Nil	Flocculent precipitate dissolves in excess reagent	Precipitate	Precipitate	Fine opaque nonsettling precipitate
Ghatti	Fine precipitate	Nil	Nil	Nil	Precipitate	Fine precipitate, nonsettling (2–3 vol.)
Gelatin	Precipitate in alkaline medium (22)	Precipitate	Nil	Nil	Nil	Finely flocculent precipitate, coagulates

[a] Rodalon (alkyl dimethyl benzyl ammonium chloride), Fairfield Laboratories, Plainfield, N. J.
[b] Mercury dissolved in twice its weight of concentrated nitric acid and diluted to 100 times its volume with distilled water.
[c] Precipitates with papain and gelatin are observed only in weakly acidic medium and most exhibit properties of coacervates rather than true precipitates.

pH of the mixture is below the isoelectric point of the protein and it is possible that they would be more correctly called coacervates. They are usually dispersed by a few drops of mineral acid or of dilute ammonium hydroxide. The characteristic manner in which some of the gums are precipitated by alcohol may also be of value in their identification.

The reactions described in Table IV form the basis of a proposed procedure for the systematic identification of the gums.

REAGENTS

Calcium chloride ($CaCl_2$), 3% solution (weight/volume).
Ammonium hydroxide, 3.0 *N* solution.
Sodium hydroxide, 3.0 *N* solution.
Barium hydroxide, saturated solution stored in a bottle equipped with a siphon and a soda-lime tube.
Basic lead acetate, 20% suspension (weight/volume). Heat to boiling, cool, and use supernatant solution.
Hydrochloric acid (or other mineral acid), 3.0 *N* solution.
Methylene blue, 0.1% aqueous solution.
Tincture of iodine (U.S.P., *14*).
Iodine-potassium iodide stock solution, containing 0.5% iodine and 1.0% potassium iodide. Iodine-potassium iodide test solution, consisting of stock solution diluted 1 to 5.
Cupric sulfate ($CuSO_4 \cdot 5H_2O$), 15% solution (weight/volume).
Borax ($Na_2B_4O_7 \cdot 10H_2O$), 4% solution.
Ruthenium red test solution (*8*).
Picric acid, saturated aqueous solution.

IDENTIFICATION PROCEDURE

Wet a 0.25- to 0.5 gram sample of the material to be identified with 1 to 2 ml. of 95% alcohol and add 50 ml. of distilled water. Suspend the solid material in the water by shaking or stirring. Heat the suspension, with frequent shaking, on a hot plate or over a burner. If the sample dissolves, discontinue heating immediately; otherwise hold at 85° to 95° C. for 15 minutes.

Group A. I. Treat a 3- to 5-ml. aliquot of the solution with 0.2 volume of 0.25 *M* calcium chloride. A gelatinous precipitate or gel indicates alginates or de-esterified pectin.

II. If either test in A I was positive, mix a fresh 3- to 5-ml. aliquot of sample with 1 volume of 3.0 *N* sodium hydroxide. Observe the reaction and then heat the mixture in a boiling water bath for 10 minutes.

Immediate formation, in the cold, of a gelatinous or flocculent precipitate indicates either pectin or de-esterified pectin. No precipitate indicates alginates. All three mixtures become yellow on heating, but the precipitates with pectic substances do not dissolve.

Group B. If the material is not an alginate or a pectic substance, carry out the following tests.

I. Mix a 3- to 5- ml. aliquot of the sample with 0.1 volume of saturated barium hydroxide. Observe in the cold and heat in boiling water bath for 10 minutes.

Formation in the cold of a nonsettling, almost opaque, gelatinous precipitate indicates Irish moss. Carry out confirmatory test for Irish moss.

A small amount of flocculent precipitate or cloudiness in the cold and a definite lemon yellow color on heating identify gum tragacanth.

Color changing during heating to yellow, then to green, and finally to gray indicates agar. Carry out confirmatory test for agar.

If the mixture becomes cloudy or forms a gel on heating, but becomes clear on cooling, methylcellulose is indicated. Carry out confirmatory test for methylcellulose.

An opaque flocculent precipitate which may tend to redisperse on heating and reprecipitate on cooling indicates starch. Carry out confirmatory test for starch.

Precipitates which disappear when the barium hydroxide is thoroughly mixed with the sample may be disregarded at this point.

II. If the material has not been identified, mix a fresh 3- to 5-ml. aliquot of sample with 1 volume of saturated barium hydroxide. Observe whether there is an immediate [illegible]

Table IV. Precipitation Reactions Used as Basis of Proposed Procedure for Identification of Gums

(Reactions used as identification tests within heavy lines)

Gum	1/5 Vol. 2.5% $CaCl_2$	1/5 Vol. 2.5% $CaCl_2$ + 1/5 Vol. 3 N NH_4OH	1 Vol. 3 N NaOH	1/15 Vol. Saturated $Ba(OH)_2$ Cold	1/15 Vol. Saturated $Ba(OH)_2$ Heated	1 Vol. Saturated $Ba(OH)_2$	1/5 Vol. Basic Lead Acetate	Basic Lead Acetate + 1/5 Vol. 3 N NH_4OH	Confirmatory Tests
[illegible]-esterified pectin	Gelatinous ppt. or gel		Gelatinous or flocculent ppt., yellow color on heating	Gel or gelatinous ppt.	Ppt. and soln. turn yellow	As for 1/15 vol.	Gels	As for lead acetate alone	Gelatinous ppt. when acidified with mineral acids
[illegible]inate	Gelatinous ppt. or gel		Clear yellow soln. on heating	Gel or gelatinous ppt.	Ppt. and soln. turn yellow	As for 1/15 vol.	Gels	As for lead acetate alone	Gelatinous ppt. when acidified with mineral acids
[illegible]tin		Gelatinous ppt. forms slowly	Gelatinous or flocculent ppt., yellow color on heating	Gel or gelatinous ppt.	Ppt. and soln. turn yellow	As for 1/15 vol.	Translucent gel	As for lead acetate alone	
[illegible] moss	Some samples may give flocculent ppt.			Gelatinous ppt. or gel, almost opaque	Ppt. may flocculate with prolonged heating	Gelatinous ppt. or gel	Gelatinous ppt. or gel	As for lead acetate alone	Forms blue fibrous ppt. with aqueous methylene blue
[illegible]racanth				May be small amount of ppt.	Lemon yellow color	Flocculent ppt.	Flocculent ppt.	As for lead acetate alone	
[illegible]				Nil	Becomes yellow then green and gray	Nil	Flocculent ppt.	Gels	Gives blue or black stain with tincture of iodine
[illegible]thylcellulose				Nil	Becomes turbid or forms gel; becomes clear on cooling	Nil	Nil	Gels	Aqueous dispersions are not precipitated by alcohol, but form gel or become cloudy when heated
[illegible]h				Opaque flocculent ppt.	May redisperse	Flocculent ppt.	Flocculent ppt.	Very heavy flocculent or gelatinous ppt.	Blue stain with I-KI reagent
[illegible]oxymethylcellulose				Ppt. dissolves on shaking	Nil	Flocculent ppt.	Gels	As for lead acetate alone	Forms clotted ppt. with $CuSO_4$
[illegible]st*						Clotted opaque ppt.	Opaque gel	As for lead acetate alone	Gels with 1/5 vol. 4% borax
[illegible]ya						Flocculent ppt., forms slowly	Flocculent ppt.	As for lead acetate alone	Swells and stains pink with ruthenium red test solution
[illegible]ic (acacia)							Voluminous opaque ppt.	As for lead acetate alone	Readily soluble in water at room temperature
[illegible]ti							May be small amount of flocculent ppt.	Voluminous opaque ppt.	Fine ppt. with 4 vol. of alcohol. Arabic also gives fine ppt.
[illegible]in									Gives fine yellow ppt. when added to saturated picric acid

*Locust bean gum and guar gum give identical reactions and cannot be distinguished on basis of these tests.

Table V. Samples Examined by Proposed Identification Procedure

Gum	Type	No. of Samples
Sodium pectate	Powdered	1
Pectic acid	Granular	1
Pectin	Powdered	2
Sodium alginate	Powdered	5
Irish moss	Powdered	2
Tragacanth	Powdered	1
Agar	Shredded	1
	Granular	3
Methylcellulose	Fibrous	2
Starch	Whole wheat flour	1
	Tapioca flour	1
	Soluble starch	3
	Cornstarch	1
	Amioca (amylopectin)[a]	1
	Clear jel[a]	1
	Clear-Flo-H (sodium salt of starch acid ester)[a]	1
	Dry-Flo (starch ester)[a]	1
	Vulca (starch ether)[a]	1
	Melojel[a]	1
	Nu-film (starch acid ester)[a]	1
Sodium carboxymethylcellulose	Powdered	1
Locust bean (carob)	Powdered	7
Guar	Powdered	1
Karaya	Powdered	1
Arabic (acacia)	Powdered	3
	Lump	1
Ghatti	Powdered	1
	Lump	1
Gelatin	Granular	2

[a] Products of National Starch Products, New York, N. Y.

A voluminous opaque, stringy precipitate which tends to form a clot indicates locust bean gum. This precipitate may appear flocculent if the mixture is shaken vigorously. Carry out confirmatory test for locust bean gum.

A voluminous opaque flocculent precipitate which forms immediately indicates carboxymethylcellulose. Carry out confirmatory test for carboxymethylcellulose.

An opaque flocculent precipitate which forms slowly and is not voluminous indicates gum karaya. Carry out confirmatory test for karaya.

Group C. If the sample has not been identified it may be gum arabic, gum ghatti, or gelatin.

I. Mix a fresh 3- to 5-ml. aliquot of sample with 1 ml. of basic lead acetate solution. Immediate formation of a voluminous opaque precipitate indicates gum arabic.

If there was only a small amount of flocculent precipitate, or no precipitate, with the basic lead acetate add 1 ml. of 3.0 *N* ammonium hydroxide to the lead-containing sample. A voluminous opaque flocculent precipitate indicates gum ghatti. If there is no precipitate, the sample probably is gelatin. Carry out confirmatory test for gelatin.

CONFIRMATORY TESTS

Alginates and De-esterified Pectins. Add 0.2 volume of 3 *N* hydrochloric acid (or other mineral acid) to 3 to 5 ml. of the sample. A gelatinous precipitate confirms alginates or de-esterified pectin.

Irish Moss. Add 2 to 3 drops of 0.5% methylene blue in water to 1 ml. of the sample solution. Precipitation of purple-stained fibers confirms Irish moss.

Methylcellulose. Mix 5 ml. of sample with 25 ml. of 95% alcohol and 2 to 3 drops of saturated sodium chloride. No precipitate confirms methylcellulose.

Agar. Precipitate gum from 5 ml. of sample with alcohol and stain with tincture of iodine (*3*). Starch is also stained blue by this reagent.

Starch. Add 1 to 2 drops of the iodine potassium iodide test solution to 1 ml. of sample. A blue or purple color confirms starch. Some samples of gum tragacanth may give a faint blue test here.

Carboxymethylcellulose. Add 2 ml. of 1.0 *M* cupric sulfate to 3 to 5 ml. of sample solution. An opaque, slightly bluish, clotted precipitate confirms carboxymethylcellulose.

Locust Bean Gum. Add 1 ml. of 4% borax to 3 to 5 ml. of gum solution. If mixture gelatinizes, locust bean gum is confirmed. Guar gum also forms a gel here.

Karaya. Precipitate gum from 5 ml. of solution with alcohol and stain with ruthenium red (*3*). If sample swells considerably and is stained pink, karaya is confirmed.

Gelatin. Add 2 to 3 drops of gum solution to 2 ml. of saturated picric acid. A fine yellow precipitate confirms gelatin.

DISCUSSION

The proposed procedure has been tested with the gums listed in Table V. It was possible to identify the modified starches as starch products, because the solubility of all these materials is decreased by barium hydroxide and all give positive tests with the iodine-potassium iodide reagent. It is possible that not all the thickening agents or gums employed in food products at the present time have been included in this study. Cherry gum and quince seed gum, for example, have been suggested in the literature for use in foods. However, the proposed procedure includes all the gums that were available during the investigation.

The proposed scheme for identification of stabilizing and thickening agents is applicable only when they have not been mixed with other materials. To identify thickening agents in foods by this method it would first be necessary to separate them from the foods, but separation techniques might alter the reaction characteristics of the gums. Much work has been done on methods for the separation and detection of gums in particular foods, such as mayonnaise and French dressing (*2, 7, 10, 11*), soft curd cheese (*1, 8, 10, 11, 13, 19*), tomato products (*10, 13*), starchy foods (*21*), cacao products (*13, 17, 23*), ice cream and frozen desserts (*9–11*), canned chicken (*10*), and meat products (*12*). Additional references to methods for separating gums from foods may be found in reviews by Jacobs and Jaffe (*15*) and Mantell (*16*). The emphasis in most procedures has been on detection of the gums without identification. However, Wyler (*24*) has outlined methods for the detection and identification of locust (carob) bean gum, methylcellulose, carboxymethylcellulose, starch, pectin, and alginate in meat products. Thus identification tests proposed in the present paper may be useful for the identification of gums separated from foods by methods already described in the literature. However, it is probable that many special techniques will be required for the analyses of particular combinations of foods and thickeners. A great deal more work must be done before it will be possible to identify all of the gums in the various foods in which they may be used.

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Amer. J. Physiol. 188(3) 1957

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Effects of Feeding Diets Containing Lactose, Agar, Cellulose, Raw Potato Starch or Arabinose on the Dry Weights of Cleaned Gastrointestinal Tract Organs in the Rat

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ABSTRACT

FISCHER, JESSIE E. (Ohio Agricultural Exp. Station, Columbus.) *Effects of feeding diets containing lactose, agar, cellulose, raw potato starch or arabinose on the dry weights of cleaned gastrointestinal tract organs in the rat.* Am. J. Physiol. 188(3): 550–554. 1957.—Purified diets containing 25% lactose, agar, wood cellulose or raw potato starch, or 10% arabinose were compared with a control diet containing cornstarch as the only carbohydrate for effects upon dry weights of cleaned gastrointestinal organs in rats. Many effects of diets upon absolute organ weights and upon organ weights relative to body weight were found. When the lactose diet was fed for 1 week from weaning, the relative small intestine weight and the absolute and relative weights of both the cecum and the colon plus rectum were increased over the controls. When all of the diets were fed for 4–5 weeks, organ weights were increased by diets as follows: relative stomach weight by agar and cellulose, absolute and relative small intestine weights by lactose, relative small intestine weight by arabinose, absolute and relative cecum weights by all experimental diets except cellulose, and absolute and relative colon plus rectum weights by all five experimental diets. Thus, stomach growth is affected by diet little or not at all, while intestine growth is differently affected in its different regions by various diets. Increases in dry weights or numbers of fecal pellets were not at all related to increases in small intestine or cecum weight, and were not well related to increases in colon plus rectum weight. Using these facts and knowledge of certain properties of the various substances, certain ideas as to modes of action of diets in producing the observed changes were excluded.

CERTAIN substances or variations in the diet stimulate the growth of various parts of the gastrointestinal tract in mammals. In rats, agar stimulates growth of the large intestine but not the small intestine (1), raw potato starch and lactose stimulate growth of the cecum (2), and lactose (3) and certain multiple variations in diet (1) stimulate growth of the small intestine. In mice, talc stimulates growth of the small intestine (4). The aims of the present work were to extend the observations with some of these same substances to other regions of the gastrointestinal tract and to shorter feeding periods, to study other substances for possible effects on gastrointestinal tract growth, and to try to find out how the various substances in the diet produced the effects on different regions of the tract.

Received for publication August 22, 1956.

[1] Approved for publication as journal article no. 57-56 by the Associate Director of the Ohio Agricultural Experiment Station.

MATERIALS AND METHODS

Animals. Weanling male Sprague-Dawley rats were divided into eight groups of six rats each. The rats were housed in individual cages which had wire mesh floors. They were fed tap water and their respective diets ad libitum until they were killed for the determinations of organ weights. All rats in *groups 1a* and *2a* were killed after 1 week of feeding. The rats in *groups 1* through *6* were killed starting after 4 weeks of feeding, and finishing 6 days later; on each working day, one rat from each of the six groups was killed. The rats were weighed at the start of the feeding period, at the end of each week, and on the day they were killed

Diets. The basal control diet was fed to *groups 1* and *1a*. It contained 67% powdered cornstarch (Buffalo brand no. 3401),[2] 24% 'vitamin-free' casein (Nutritional Biochemicals Corp.), 5% Crisco, and 4% salt mixture, U. S. P. XIV. Vitamins were added to the diet at the levels previously given and halibut liver oil was given individually as before (3). For the experimental diets, the test substance replaced an equal weight of cornstarch in the basal diet. The experimental dietary groups and the materials incorporated into the diets were as follows: *groups 2* and *2a*, 25% lactose, U. S. P. (Merck); *group 3*, 25% ball-milled agar (Bacto-Difco); *group 4*, 25% ground wood cellulose (Alphacel, Nutritional Biochemicals Corp.); *group 5*, 25% raw potato starch; *group 6*, 10% L(+)-arabinose (Nutritional Biochemicals Corp.). Arabinose was chosen for study because it is quite slowly absorbed and causes diarrhea in the rat (5, 6), and thus might be expected to have effects on intestinal growth somewhat similar to those of lactose, the other diarrhea-producing sugar (7) in the study. The dietary level of arabinose was set at only 10% to keep well below the 35% level known to be lethal to rats (6).

Feces. During the feeding period, daily records were made of any diarrhea which occurred.

On the 5th day of the 4th week of the feeding period, a 24-hour feces collection was made from each rat. The number of fecal pellets was counted. Then the feces were placed in tared weighing bottles and dried over sulfuric acid in a vacuum desiccator at room temperature. The bottles were weighed at weekly intervals with recharging of the desiccator each time it was opened, until the additional weekly weight loss decreased to 7 mg or less. This was considered close enough to constant weight for the purposes of this experiment. The drying process required 8 weeks for the feces from the agar-fed group and 3 weeks for the feces from all other groups. The average dry weight per pellet was calculated by dividing the total dry weight of the collection from each rat by the number of pellets in the collection. No attempt was made to obtain the weight of the freshly-voided feces.

Dry Weights of Cleaned Organs. The rats were stunned and bled. The alimentary tube was exposed, and the mesenteries were stripped cleanly off it from the cardia to the anus. The stomach, small intestine, cecum and colon plus rectum were removed separately into beakers of ice-cold distilled water, and slit open lengthwise. The contents of each organ were removed in the water by gently brushing with a small paintbrush and by rinsing up and down in beakers of clean water. The thoroughly cleaned organs were blotted gently on filter paper and examined. Then they were put into tared weighing bottles, dried overnight at 100°C in a gravity convection oven, cooled and weighed. The relative organ dry weights in milligrams per 100 gm of live body weight were calculated, but are not given in the table. However, differences in relative organ weights as well as differences in absolute weights (which are given in the table) were analyzed statistically and are noted in the results and discussion.

[2] This product was the generous gift of the Corn Products Sales Co., New York, N. Y.

TABLE 1. AVERAGE BODY WEIGHT GAINS OF RATS FED SIX DIFFERENT DIETS AD LIBITUM

Dietary Group	Gains in Successive Weeks, gm	Total Gain in 4 Weeks, gm
Cornstarch, *1a*	38	
Lactose, *2a*	30	
Cornstarch, *1*	36, 49, 44, 40	170
Lactose, *2*	32, 45, 43, 41	160*
Agar, *3*	41, 49, 37,† 35	162
Cellulose, *4*	37, 45, 37,† 32†	151†
Potato starch, *5*	39, 53, 39, 38	169
Arabinose, *6*	32, 47, 42, 40	161

Five rats in the potato starch group; six rats in each other group. Average body weight at the start in each group was 46 gm.

In the statistical comparison of quantities from each experimental group with the corresponding quantities from the corresponding cornstarch control group, the alternative to the null hypothesis was that each experimental group was not equal to the control group except where marked differently.

* The alternative was that the cornstarch control exceeded the experimental.

† $P < 0.05$.

Statistical Treatment of Data. The 'U' test (8) was used for the statistical analysis of differences.[3] The hypothesis tested for each quantity was that there was no difference between each experimental group and the control (cornstarch diet) group. The alternative for each of the comparisons is given in the tables.

RESULTS

Body Weight Gains (Table 1). Differences in gains which were statistically significantly different from the control group were found only in the agar group during the 3rd week of the feeding period, and in the cellulose group during the 3rd and 4th weeks and for the total 4 weeks feeding period. In each of these instances, the gains were less in the experimental group than in the control group.[4] One rat in the potato starch group died dur-

[3] Statistical analysis was kindly carried out at the Statistics Laboratory, The Ohio State University, Columbus, Ohio.

[4] It will be noted later in table 3 that there are no significant differences in body weight on the killing day when each of the experimental groups is compared to the control group. In table 1, however, the difference in the 4-wk. gain between cellulose *group 4* and control *group 1* is significant. This apparent discrepancy resulted from the procedure of using the rats at the rate of 6/day, while allowing the remaining rats to continue on the diets until all were used. Differences in additional weight gains made during the few days beyond 4 wk. narrowed the body weight difference between *groups 1* and *4* so that the difference no longer was significant statistically.

TABLE 2. NUMBER OF FECAL PELLETS AND DRY WEIGHT OF FECES

Dietary Group	No. of Pellets	Dry Weight	
		Total feces	Average/pellet
		gm	*mg*
Cornstarch, *1*	10 (6–16)	0.37 (0.23–0.54)	36 (30–39)
Lactose, *2*	12 (7–22)	0.56 (0.39–0.94)	46 (39–59)†
Agar, *3*	66 (56–77)†	5.05 (4.65–5.85)†	78 (66–86)†
Cellulose, *4*	42 (32–48)†	5.38 (4.39–6.31)†	130 (118–143)†
Potato starch,* *5*	18 (11–22)	1.73 (1.03–2.64)†‡	100 (52–155)†
Arabinose, *6*	9 (7–13)	0.44 (0.20–0.83)	47 (29–64)

Data from one 24-hour feces collection made the 5th day of the 4th week of the feeding period. Four rats in the potato starch group; six rats in each of the other dietary groups. Figures given are averages; ranges in parentheses.

* Data were obtained also from a fifth rat which exhibited diarrhea on the day of the feces collection. The feces from this rat consisted of 23 well-formed pellets plus 24 deformed or fluid discharges; the total dry matter in the collection was 2.71 gm. These data are not included in the averages or ranges.

† $P < 0.01$.

‡ The alternative to the null hypothesis was that the cornstarch control was less than the experimental. In all of the other comparisons in this table the alternative was that each experimental group was not equal to the cornstarch group.

ing the 3rd week of the experiment. The cause of death could not be related definitely to the diet fed. Gain data from this rat are not included in the table.

Observations and Measurements on Feces. Diarrhea was observed in only one rat of the corn starch group during part of the 1st week only. Diarrhea never occurred in the agar or cellulose groups. Only a little diarrhea was seen in the raw potato starch group in the first 2 weeks of the feeding period, but the frequency of occurrence and severity of the diarrhea increased during the 3rd and 4th weeks on this diet. Rather severe and frequently occurring diarrhea was observed in the lactose and arabinose groups during the first 2 weeks of feeding; after this the diarrhea was less severe and occurred only infrequently in these two groups.

The numbers of fecal pellets and the dry weights of the feces are given in table 2. The numbers of fecal pellets in the 24-hour collection were significantly increased in the agar and cellulose groups. The total dry weight of the feces was significantly increased in three of the experimental groups; the increase was about 13-fold in the agar and cellulose groups and about 5-fold in the raw potato starch group. The average dry weight per pellet was increased significantly in all experimental groups except for the arabinose group. Some increase in weight/pellet occurred with arabinose also such that the average magnitude of this quantity was about the same as the average in the lactose group; however, the difference did not prove to be significant by the statistical test used.

Organ Weights After 1 Week of Feeding (Table 3). The lactose diet only was tested at this time interval. The stomach weight was not changed by lactose feeding. The small intestine weight was increased significantly on the relative weight basis only. The weights of both the cecum and the colon plus rectum were significantly increased both absolutely and relatively. The change in the cecum was especially striking. These changes in the cecum and the colon plus rectum in the growing rat after only 1 week on the lactose diet show the speed with which these two regions may respond to dietary stimulation.

Organ Weights After 4–5 Weeks of Feeding (Table 3). All five experimental diets were tested at this time interval. The stomach weight was increased only by agar or cellulose feeding; these increases were significant only on the relative basis. The small intestine weight was increased both absolutely and relatively by lactose, and relatively by arabinose. The absolute small intestine weight in the cellulose-fed rats was less than in the controls, but the relative weights were not different. The cecum weight was increased both absolutely and relatively by all of the test substances except cellulose. The magnitudes of the cecum weight increases ranged from greatest to least as follows: raw potato starch > arabinose > lactose > agar. The weight of the colon plus rectum was increased both absolutely and relatively by all five test substances, showing the susceptibility of this region to dietary stimulation. The magnitudes

of the colon plus rectum weight increases ranged from greatest to least as follows: agar > raw potato starch and cellulose > arabinose and lactose.

Appearance of Organs. In the groups in which the cecum dry weight was increased it appeared that the cecum after cleaning was a bigger sac rather than an obviously thicker-walled sac. The heavier colons, on the other hand, felt definitely thicker, especially in the agar group. In one rat of the six in the cellulose group, a fistula was found in the body of the stomach along the greater curvature. In one rat of the six in the agar group and in all six of the rats in the cellulose group, red transverse stripes were seen in the terminal half of the colon. The stripes were not changed much in appearance by brushing the mucosal or serosal surfaces with the moistened paintbrush, so apparently they did not represent solely surface bleeding. They appeared to be buried in, or to extend into, some deeper layer(s) of the colon. Blood was present on the surface of some of the pellets removed from the colon of the cellulose-fed rats.

TABLE 3. AVERAGE BODY WEIGHT AND WEIGHTS OF CLEANED DRIED ORGANS

Dietary Group	Body Weight	Weights of Cleaned, Dried Organs			
		Stomach	Small intestine	Cecum	Colon + rectum
	gm	*mg*	*mg*	*mg*	*mg*
Killed after 1 week on diets					
Cornstarch, *1a*	84	111	572	39	89
Lactose, *2a*	76	107	598†	84†§	98‡
Killed after 4-5 weeks on diets					
Cornstarch, *1*	236	235	1043	81	147
Lactose, *2*	219*	213	1170†‡	159†§	169‡
Agar, *3*	220	256	962	111§	323§
Cellulose, *4*	210	239	948‡	78	247§
Potato starch, *5*	226	219	1009	290†§	254§
Arabinose, *6*	222	218	1144	231§	182§

Five rats in the potato starch group; six rats in each of the other groups.

In the statistical comparison of quantities from each experimental group with the corresponding quantities from the corresponding cornstarch control group, the alternative to the null hypotheses was that each experimental group was not equal to the control group, except where marked differently.

* The alternative was that the cornstarch control group exceeded the experimental group.

† The alternative was that the cornstarch control group was less than the experimental group.

‡ $P < 0.05$. § $P < 0.01$.

DISCUSSION

Body Weight Gains. The ability of weanling rats to gain quite well when fed the agar, cellulose, raw potato starch or arabinose diets demonstrates a significant fact. This is that the relative indigestibility or unabsorbability of these materials at the levels fed need not necessarily impose any serious barrier to rapid gaining when ad libitum feeding is employed. This is similar to the situation in mice fed 50% talc in the diet (4), where gains were equal to the gains of control mice fed a diet devoid of this indigestible material.

Feces. The quantitative changes in the feces showed fairly characteristic patterns for some of the diets. Thus, while both the number of fecal pellets and the dry weight per pellet increased strikingly on both agar and cellulose diets, these two diets differed in the amount of change in each of these quantities. Again, the dry weight per pellet increased markedly on the raw potato starch diet and slightly on the lactose diet, while the number of pellets changed little or not at all. The patterns of fecal excretion and the known physical and chemical properties of the diet constituents were considered in relation to the increases in dry weights of the organs. This approach enabled us to eliminate certain ideas as to how diet substances might increase intestinal growth, as will be shown below for each of the regions of the intestine.

Stomach Weights. Judging from the present work with six diets, it appears likely that any dietary effect on the shrinking or expanding of the stomach in well-fed rats of the same size and age involves almost exclusively contraction or distention, not atrophy or growth. This situation in the stomach contrasts with the findings of definite growth-stimulatory effects of diet upon several regions of the intestine.

Small Intestine Weights. The finding of a greater dry weight (both absolute and relative) of the small intestine in the lactose-fed rats after 4-5 weeks of feeding confirms by still another criterion previous work wherein small intestine growth (by fresh weight and total nitrogen) was stimulated by lactose (3). The increase in small intestine dry weight in the present work after 1 week on the lactose diet was not so definite as at 4-5 weeks, since

only the relative weight increase was statistically significant.

Besides lactose, only arabinose had any stimulating effect on small intestine growth. While this effect was statistically significant on the relative weight basis only, the absolute difference also was close to statistical significance ($P = 0.079$). If, in the statistical analysis, it had been possible to use the same alternative to the null hypothesis for the arabinose comparison as was used for the lactose comparison, then the absolute difference for arabinose also would have been statistically significant (*i.e.*, P would have been $= 0.04$). We consider the small intestine weight increase on the arabinose diet to be physiologically significant.

Besides lactose and arabinose, the other substance which has been shown to stimulate small intestine growth is talc (4). The mode of action of these substances is not known. However, the present results show that indigestibility of itself cannot be an important factor in small intestine growth stimulation, since agar, cellulose, and raw potato starch do not increase growth in this organ. Another argument against the possibility that a bulk action is involved is that both lactose and talc stimulate the growth of the mucosa rather than the muscle of the small intestine (3, 4). The possibility that an indirect, hormone-mediated action is involved in producing the mucosal growth increases was discussed elsewhere (3).

Cecum Weights. The lack of any effect of the 25% wood cellulose diet on cecum weight is reminiscent of results with 6% rice cellulose in the diet (2). Also, the increase in weight of the cecum in rats on the agar diet, while statistically significant, was relatively slight in magnitude. These results with cellulose and agar feeding, considered together with the fecal data, show that increases in the amounts of dry fecal residue are not connected with increases in cecum weight. This invalidates our earlier suggestion (2) that there might be such a connection.

The question then arises as to how dietary constituents increase the weight of the cecum. Diet may influence fluidity of the cecal contents, total amount of cecal fill, and microbiological activities in the cecum. There are ways in which changes in each of these factors conceivably could influence the growth of the cecum, but nothing very definite is known about the actual role of any of them.

Colon Plus Rectum Weights. In other work considering dietary effects on the weight of the large intestine, effects on the colon plus rectum were not reported separately from effects on the total large intestine (1, 4). The present results (especially those with cellulose, where there was no effect on the cecum and a large effect on the colon plus rectum) emphasize the value of such a separation.

The diet-produced increases in colon plus rectum weights perhaps are related to an increased amount of work done in drying especially fluid residues by the proximal colon (9) and/or in mixing, moving, shaping and expelling large amounts of residue by the entire colon. The amount of residue expelled should reflect the amount of work done in the latter of these two processes, and should therefore be related to growth in this region. To test this idea, the dietary groups were ranked according to colon plus rectum weight, and also according to number of pellets, total dry weight of feces, and average dry weight per pellet, in turn. It was found that none of the three measures of fecal output agreed completely in rank order or relative degree of change with the rank order or degree of change in the colon plus rectum weight series. Thus, while we did not discover any characteristic of the residue which was well related to the degree of increase in weight of the colon plus rectum, several characteristics were eliminated from consideration. Several other characteristics remain to be tested.

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COMMENTS ON THE MECHANISM OF THE FETOTOXIC EFFECT OF TRAGACANTH

(Über den Mechanismus der fetalschädigenden Wirkung von Traganth)

Authors: H. Frohberg, H. Oettel and H. Zeller

Source: Archiv für Toxikologie, Vol. 25, 1969, Springer-Verlag Berlin, Heidelberg, New York, P. 268-295.

A. Introduction:

Not all the substances, whose possible teratogenic effect must be tested, are soluble in water, physiological salt solution and Ringer's solution. Hence, they must be applied, in the form of suspensions or emulsions according to their mode of utilization, orally or parenterally. Natural substances, beside synthetic products like carboxymethyl cellulose, are used as suspensions in manufacturing these preparations. That is why the influence of suspensions involving natural substances was tested in comparison with distilled water and Ringer's solution as to gestation in mice. This was done especially since WILLIAMSON and Collaborators (1963), as well as CARTER (1965), proved in their tests conducted on chick embryos that inert substances, like sand, ground glass, colloidal Al or talc, produced malformations with the same frequency as definitely teratogenic substances.

B. Material and Methods:

1. Animal Species Used:

Mice of the NMRI-breed were used for the tests (animals from closed random breeding; Breeder Dr. Haakh, until late 1964: Federal Research Institution for Virus Diseases in Animals, since 1965: Firma Ivanovas, Kissleg, Allgäu). Upon reception, all the mice received a protective inoculation of active vaccine virus against ectromelia at the Industrial Hygiene Pharmacological Institute of the BASF (Zeller and Reckzeh, 1965).

2. Type of the Substances Tested and of the Substance Specimens:

Table 1 contains a survey of the substances tested and substance specimens, and reveals their origins.

Table 1: Survey of the substances tested

Bezeichnung	Chargen-nummer	Herkunft	
Aqua destillata	—		
Ringerlösung	—		
Gummi arabicum	G 1012	E. Merck, AG	Darmstadt
Agar	51581	E. Merck, AG	Darmstadt
Carrageen	—	F. Schulze u. Co	Mannheim
Guar	M 175	Meyhall Chemical AG	Kreuzlingen (Schweiz
Talkum	T 61754	E. Merck, AG	Darmstadt
persischer Traganth Nr. 0	—	E. Merck, AG	Darmstadt
persischer Traganth Nr. 1	434209	E. Merck, AG	Darmstadt
persischer Traganth Nr. 2	387011	E. Merck, AG	Darmstadt
persischer Traganth Nr. 3	263868	E. Merck, AG	Darmstadt
persischer Traganth Nr. 4	277520	E. Merck, AG	Darmstadt
persischer Traganth Nr. 5	419414	E. Merck, AG	Darmstadt
persischer Traganth Nr. 6	8021/65	Imhoff u. Stahl, GmbH	Mannheim
persischer Traganth Nr. 7	B 420/5165	Riedel de Haen, AG	Seelze-Hannover
indischer Traganth Nr. 1	14009/64	Imhoff u. Stahl, GmbH	Mannheim
indischer Traganth Nr. 2	562/65	Imhoff u. Stahl, GmbH	Mannheim

The terms "Persian tragacanth" designate the officinal tragacanths from the Near East. These are mucilages obtained mainly from Astragalus gummifer Labillardière and from related asiatic astragal species, all of them belonging to the Leguminosae family (TSCHIRSCH, 1912; BERGER, 1924). In previous time, this astragal mucus, called also "true tragacanth", was extended with "Indian tragacanth", and still today Indian tragacanth is often used as a substitute for the more expensive pure tragacanth.

Indian tragacanth, called also "false tragacanth", Karaya- or Sterculia-rubber, is obtained by scraping the bark of the trunk of Sterculia ureaus Roxb., a tree widely distributed in Hither India (Hindustan). Unlike true tragacanth, Sterculia-rubber contains no starch, possesses no adhesive power, and swells much more rapidly in water.

3. Nutrient Media:

Merck-Standard I-nutrient agar and Merck-Standard-nutrient broth were used in obtaining the bacteriological nutrient media. The various differential nutrient media were prepared according to the prescriptions indicated by HALLMANN ("Bakteriologische Nährböden" - Bacteriological Nutrient Media, Thieme-Verlag, Stuttgart, 1953).

4. Methode Used in Testing the Teratogenic Effect:

In teratogenic tests, the fertilization periods should be indicated as accurately as possible. Hence, in our Institute, when teratogenic tests are conducted on mice, several 100 pubescent female mice are placed together each time for 2 hours with the males (bucks) (5 females with one male), once a week from 10 to 12:00 AM. When the cohabitation is terminated, each animal exhibiting a vaginal plug is isolated. In the case of these "plug mices", the pairing day is called the 1st day of gestation (FROHBERG and OETTEL, 1964).

On the 19th day of gestation, thus one day before the normal littering day, all the treated or untreated mice are killed, and the number of the implantation places, of the intact and resorbed fetuses, as well as the number of the dead but externally uninjured fetuses, are determined. The implantation places, which can be recognized by the "metrial glands", which can be identified as quite perfused yellowish nodules on the mesometrium insertion of the uterine excrescences, and which are not occupied by living or artificially dead fetuses or fetal resorptions, characterize the number of the miscarried embryos.

The body length and weight of the fetuses are determined, then their malformations are examined macroscopically. This examination is conducted systematically from head to tail. Subsequently, the fetuses are fixed in alcohol; the soft parts are clarified in caustic potash and, in order to evaluate the individual bones, the skeletal system is dyed with alizarin red S by a modified Dawson method. These clarified and colored fetuses are kept in 100% glycerine DAB 6 (FROHBERG and OETTEL, 1966).

In order to be able to make a statement regarding the possible embryotoxic effect of a product in the case of the animal species used for the tests, one must possess an accurate knowledge of the spontaneous values. Hence investigations of the littering size, of the rates of fetal resorption and of malformation, were conducted on 413 pregnant and untreated NMRI mice.

In the uteri of 414 untreated animals, 4293 implantation places were found, that is 10.4 on an average per mother animal (See Table 2).

Table 2: Spontaneous values in 414 NMRI control-mice. The mother animals were caused to die on the 19th day of gestation.
a. Implantations b. Fetuses c. Alive d. Malformed e. Dead
f. Resorptions g. Miscarriages h. Total number i. Average per animal

	a. Implantationen	b. Feten lebend	c. mißgebildet	d. runts	e. tot	f. Resorptionen	g. Aborte
Gesamtzahl (h)	4293	3918	60	18	20	343	12
Durchschnitt pro Tier (i)	10,4	9,5	0,14	0,04	0,05	0,83	0,03

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From the 4293 implanted embryos, 343 had died or had been resorbed. Thus the average quota of fetal resorption per animal was 0.83- One must bear in mind, however, that fetal resorptions were found only in 199 of the 414 control mice, and also one resorption in 120 animals, 2 resorptions in 44 mice, and more than 2 resorptions in 35 animals. One mouse, in each case, had even 6 or 8 spontaneous resorptions. On the 11th day of gestation, in a control animal, all the 12 fetuses were expelled as miscarriages.

For the most part, the mice had 9 - 13 fetuses per litter, and the 3918 living fetuses of the 414 control animals exhibited on an average a length of 2.2 cm and a body weight of 1.2 g. Eighteen living fetuses of the altogether 14 mother animals, must be regarded, with a body weight of only 0.6 - 0.8 g, as "congenital runts". In 18 mothers, altogether 20 dead, nonmalformed, "normally" developed fetuses were found in the uterus.

Figure 3: Spontaneous malformations in 3918 fetuses from 414 NMRI-control-mice. The mother animals were caused to die on the 19th day of gestation.
a. Type of malformation b. Number of malformed fetuses c. Frequency
d. Cleft palates e. Thoracic vertebral body hyplasty g. Aplasia of the vertebrae and ribs h. Malformations of the ribs i. Out of 50 mother animals.
f. execephaly. j. microcephaly k. micronathy

a. Art der Mißbildung	b. Zahl der mißgebildeten Feten	c. Häufigkeit (%)
d. Gaumenspalten	40	1,02
f. Exencephalie	6	0,15
j. Mikrocephalie	2	0,05
k. Mikrognathie	1	0,03
e. Brustwirbelkörperhypoplasie	4	0,10
g. Wirbel- und Rippenaplasie	1	0,03
h. Rippenmißbildungen	6	0,15
	60	1,53
	i. von 50 Muttertieren	

Out of the 3918 living fetuses, 60 (1.53%), which originated from 50 (12%) of the 414 mother animals, exhibited spontaneous malformations (Table 3), and also 40 fetuses (1%) had cleft palates. Thus they presented the most frequent spontaneous malformation in our mice-breed. Four fetuses had execephaly, 2 fetuses had microcephaly, 1 fetus exhibited a micrognathy, and in 4 fetuses, hypoplasiae or aplasiae of some thoracic vertebrae were detected. Six fetuses had malformations of the ribs, and in one stunted animal, all the thoracic vertabrae, the ribs, and most of the lumber vertebrae were missing.

C. Results:

1. Testing the Fetotoxic Effect of Different Suspensions:

From the 11th to the 15th day of pregnancy, gravid mother-mice received daily 0.2 ml per animal of the suspension preparations (mentioned in Table 4) by intraperitoneal injection. The individual results are shown in Table 4.

Table 4: Effects of suspensions on the pregnancy of NMRI mice 5 x 0.2 ml/animal intraperitoneally as aqueous suspension from the 11th to the 15th day of gestation (The mother animals were caused to die on the 19th day of gestation)

a. Substance b. Number of mother animals c. Total number per group with miscarried fetuses d. Implantations e. Total number per group f. Average per mother animal g. Resorptions h. % referred to the total number of implantations i. Miscarriages j. Fetuses k. Alive l. Weight m. Length n. Dead o. Malformed p. Type of malformation q. Distilled water r. Ringer's solution I. Cleft palate 2. Umbilical hernia 3. Malformation of the ribs 4. Aplasia of the Os interparietale.

a. Substanz	b. Muttertierzahl		d. Implantationen		g. Resorptionen		i. Aborte		j. Feten							
									k. lebend				n. tot	o. mißgebildet		
	c. ges.	mit mißgeb. Feten	ges.	0	h. ges.	%	h. ges.	%	h. ges.	0	l. Gewicht g 0	m. Länge cm 0	ges.	ges.	%	p. Art der Mißbildung
q. Aqua dest.	21	1	225	10,7	24	10,7	0	0	200	9,5	1,2	2,2	1	1	0,4	1 × 1
r. Ringerlösung	5	0	54	10,8	4	7,4	0	0	50	10,0	1,3	2,5	0	0	0	—
Gummi arabicum (1%)	8	1	92	11,5	3	3,3	0	0	88	11,0	1,3	2,2	1	1	1,1	1 × 2
Agar (1%)	9	2	94	10,4	9	9,6	4	4,3	81	9,0	1,2	2,2	0	4	4,3	3 × 1 1 × 3
Carrageen (1%)	9	1	92	10,2	9	9,8	0	0	82	9,1	1,2	2,2	1	1	1,1	1 × 1
Guar (1%)	11	0	113	10,3	42	37,2	9	8,0	52	4,7	1,1	2,1	10	0	0	—
Guar (1%) 5 × 0,1	8	0	72	9,0	12	16,7	2	2,8	55	6,9	1,1	2,1	3	0	0	—
Talkum (1%)	9	1	87	9,7	11	12,6	0	0	76	8,4	1,2	2,3	0	1	1,1	1 × 2
Traganth Nr. 0 (1%)	8	0	82	10,3	44	53,7	38	46,3	0	0	—	—	0	0	0	—
Gummi arabicum (10%)	18	5	202	11,2	57	28,2	0	0	145	8,1	1,2	2,3	0	6	3,0	3 × 1 3 × 4
Talkum (10%)	14	1	128	9,1	50	39,1	0	0	72	5,1	1,2	2,2	6	2	1,6	2 × 1

ges. = Gesamtzahl pro Gruppe, 0 = Durchschnitt pro Muttertier, % = bezogen auf Gesamtimplantationszahl. 1 = Gaumenspalte 2 = Umbilicalhernie, 3 = Rippenmißbildung, 4 = Aplasie des Os interparietale.

After a 5 time intraperitoneal injection from the 11th to the 15th day of gestation of each time 0.2 ml per animal of distilled water, Ringer's solution, 1% aquaous gum arabic, agar and carrageen mucilage or of a 1% aqueous talc suspension, there was no disturbance of the pregnancy. After the injection of corresponding amounts of a 1% aqueous guar mucilage, one of the 11 mother mice died intercurrently. Forty-two fetuses were resorbed, 9 were expelled as miscarriages, and 10 were dead at the time of the killing of the mother animals

on the 19th day of gestation. In doses of 5 times 0.1 ml/animal, a 1% guar mucilage no longer influenced practically the fetal development.

After the 5 time intraperitoneal injection of each time 0.2 ml of a 1% aqueous mucilage of a commercially obtained Near East DAB 6 tragacanth, surprisingly all the fetuses were resorbed or were expelled as miscarriages. This ~~embryo~~feto-toxic effect of the 1% aqueous tragacanth mucilage investigated was considerably stronger than that of a 10% aqeous gum arabic mucilage or of a 10% aqueous talc suspension.

2. Embryotoxic Effect of Persion Tragacanth:

Since the ~~embryo~~feto-toxic effect (observed in the first test) of the investigated tragacanth specimen stood in contrast with all the other suspensions tested, and in order to exclude experimental errors or an accidental contamination of this specimen by unknown active substances, which could have caused the embryotoxic effect, 5 other charges (No. 1 - 5) of the DAB 6-tragacanth (of Persion origin) provided by the Merck Company and used in Test 1, as well as in each case one specimen of Persion tragacanth of the Imhoff & Stahl Company (No. 6) and of the Riedel de Haen Company (No. 7) were introduced into the investigations.

In these tests the animals were injected in each case with 0.2 ml of the 1% mucilages in distilled water only on the 11th and 12th day of gestation. Furthermore, some mice were treated on the 14th and 15th day of gestation with three different Persion tragacanth specimens in the corresponding way. The individual results are given in Figure 1.

Figure 1: Embryotoxic effect of Persion tragacanth for NMRI mice 2 x 0.2 ml/animal intraperitoneally as 1% aqeous mucilage. Resorptions, Miscarriages, Dead fetuses, Alive fetuses.

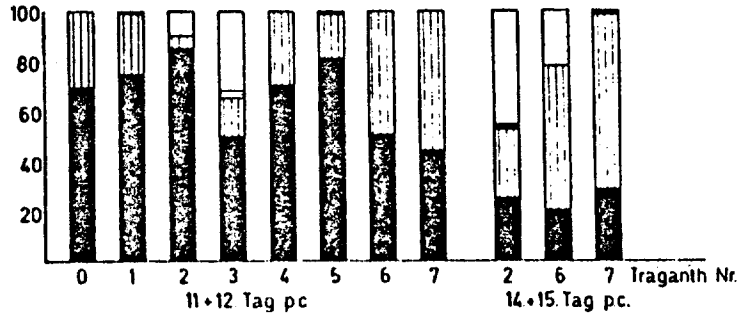


Abb. 1. Embryotoxische Wirkung persischen Traganths für NMRI-Mäuse 2 · 0,2

Five hundred and twenty eight of the 818 fetuses of the 87 mother mice, into which each time 0.2 ml/animal had been injected intraperitoneally on the 11th and 12th day of gestation, were resorbed, and 255 were expelled as miscarriages. After injection on the 14th and 15th day of pregnancy, 12 of the 160 fetuses of the 15 mother animals were dead, 40 resorbed, and 82 were expelled as miscarriages.

The miscarriages, externally recognizable through vaginal bleedings, occurred in most cases 2 to 3 days after the treatment. While animals were being killed during vaginal bleeding, dead fetus residues were found in the uteri.

Thus all the tested charges of Persian tragacanth exerted a considerable embryotoxic effect.

Since already an only two-time intraperitoneal injection of 0.2 ml each time of a 1% tragacanth mucilage of Persian origin, had a highly ~~embryo~~feto toxic effect, a Persian tragacanth specimen of the three supplying Companies was injected into gravid mice only one intraperitoneally. The injections were conducted on the 11th, 12th or 14th day of gestation. See the individual results in Table 5.

Table 5: ~~Embryo~~Feto toxic effect of Persian tragacanth for NMRI mice 1 x 0.2ml/animal intraperitoneally as 1% aqueous mucilage (The mother animals were caused to die on the 19th day of gestation)

a. Day of application percent b. Number of mother animals c. Total number per group d. with born fetuses e. Implantations f. Average per mother animal g. Resorptions h. % referred to the total number of implantations i. Miscarriages j. Fetuses k. Alive l. Weight m. Length n. Dead o. Malformed p. Type of malformation I = cleft palate 2 = Kyphosis 3 = Malformation of the thoracic vertebral body 4 = Umbilical hernia 5 = Clubfoot on the left aplasia of the metatarsals and phalanges III and IV.

Tabelle 5. *Embryotoxische Wirkung persischen Traganths für NMRI-Mäuse 1 × 0,2 ml/Tier i.p. als 1%iger wäßriger Schleim (Tötung der Muttertiere am 19. Trächtigkeitstag)*

Traganth Nr.	a Applikationstag p.c.	b Muttertierzahl		e Implantationen		g Resorptionen		Aborte		j Feten							
		c ges.	mit mißgeb. Feten	f ges.	0	f ges.	%	ges.	%	k lebend ges.	0	l Gewicht g 0	m Länge cm 0	n tot ges.	o mißgebildet ges.	%	p Art der Mißbildung
2	11	8	3	85	10,6	11	12,9	0	0	72	9,0	1,1	2,1	2	6	7,1	6 / 1
6	11	10	0	104	10,4	58	55,8	13	12,5	27	2,7	1,0	2,1	6	0	0	–
7	11	6	0	64	10,7	46	71,8	9	14,1	9	1,5	1,2	2,3	0	0	0	– –
0	12	15	5	150	10,0	45	30,0	0	0	105	7,0	1,2	2,2	0	10	6,7	9 / 1 1 · 5
2	12	11	5	116	10,5	17	14,7	5	4,3	93	8,5	1,1	2,2	1	14	12,1	13 · 1 1 · 4
6	12	8	2	81	10,1	55	67,9	0	0	22	2,8	1,0	2,0	4	3	3,7	3 · 1
7	12	8	0	84	10,5	22	26,2	62	73,8	0	0	0	0	0	0	0	– –
0	14	5	0	45	9,0	41	91,1	4	8,9	0	0	0	0	0	0	0	– –
2	14	3	2	30	10,0	5	16,7	0	0	24	8,0	1,1	2,2	1	3	10,0	3 · 1
6	14	8	2	90	11,3	20	22,2	0	0	22	2,8	1,0	2,1	48	7	7,8	5 · 1 1 · 3 1 / 2
7	14	5	0	52	10,4	0	0	31	59,6	4	0,8	1,0	2,1	17	0	0	–

ges. = Gesamtzahl pro Gruppe, 0 = Durchschnitt pro Muttertier, % bezogen auf Gesamtimplantationszahl, 1 = Gaumenspalte, 2 = Kyphose, 3 = Brustwirbelkörpermißbildung, 4 = Umbilicalhernie, 5 = Klumpfuß links Aplasie der Metatarsalia und Phalangen III und IV.

Abb. 2. Links: Fet mit hypoplastischer Fehlbildung der hinteren Extremität und Tibiaaplasie. Behandlung der Muttermaus: einmal 0,2 ml pro Tier i.p. eines 1%igen wäßrigen Schleims aus persischem Traganth am 12. Trächtigkeitstag. Rechts: Normaler Kontrollfet. Muttermäuse wurden am 19. Trächtigkeitstag getötet. Aufhellung und Anfärbung des Skeletsystems nach einer modifizierten Dawson-Methode

In these tests, the strongest ~~embryo~~feto toxic effect was exerted by Charge No. 7, but even after injection of the tragacanth-Charge No 6 on the 11th or 12th day of gestation, the majority of the fetuses were resorbed or expelled as miscarriages.

After the injection of this tragacanth (No. 6) on the 14th day of gestation, 20 fetal resorptions, 22 alive and 48 dead fetuses were found in the uteri of the 8 mother mice treated. One of the 22 living, and 4 of the 48 dead fetuses had cleft palates. Furthermore, kyphosis was detected in a dead fetus, and in another a malformation of the thoracic vertebral body. The seven malformed living and dead fetuses originated from two of the eight mother mice.

The Persian tragacanth specimens No. 0 and 2, used for these tests, produced fetal resorption and abortions with lower frequency; nevertheless, 29 of the 294 living fetuses and 2 of the dead ones had cleft palates. The 10% proportion of cleft palates is 10 times higher than the spontaneous cleft palate frequency (1.02%) of our NMRI mice-breed. The 29 living and 2 dead fetuses with cleft palates come from 14 different mother animals.

Cleft palates occurred most frequently in the mother mice treated on the 12th day of pregnancy with Persian tragacanth mucilage No. 0 and No. 2; then in 10 of the 26 mother animals, fetuses (21 living and 1 dead) were found with a fissure of the palate. Furthermore, one fetus of these 10 mother animals had an ectopia of the abdominal organs. In another fetus with uranoschism, once the skeleton had been colored, a hypoplastic malformation of the right rear extremity with tibial aplasia was detected (Figure 2). Thus altogether in 10 of the 26 mother mice treated on the 12th day of gestation, 23 living fetuses, and one dead, were found with malformations.

Figure 2: Left: Fetus with hypoplastic malformation of the read extremity and tibial aplasia. Treatment of the mother mouse: one 0.2 ml per animal intraperitoneally of a 1% aqueous mucilage from Persian tragacanth on the 12th day of gestation. Right: Normal control fetus. The mother mice were caused to die on the 19th day of pregnancy. Clarification and coloration of the skeletal system by a modified Dawson method.

A 1% aqueous mucilage of Persian tragacanth of different origin(s) exerted an embryotoxic effect not only after the repeated, but already after the single intraperitoneal injection of 0.2 ml per animal in the middle of the gestation in the case of the NMRI mice used for the investigations.

In order to determine whether tragacanth from other regions of cultivation possesses the same embryotoxic effect as Persion tragacanth, two different specimen of Indian tragacanth (No. 1 and 2) were tested in the same way as in Experiment 1.

Unlike Persian tragacanth, these Sterculia-rubber specimens, even after the five-time injection of 0.2 ml per animal from the 11th to the 15th day of gestation - a dose which in the case of the Persian tragacanth always caused 100% fetal death - failed to affect the fetal development of the mice. Only the proportion of resorptions was slightly raised as compared with the normal occurrence, and 7 of the 239 fetuses (2.9%), from 7 different mother animals, had uranochism.

The repeated five time subcutaneous injection of each time 0.2 ml per animal from the 11th to the 15th day of gestation of a 1% aqueous mucilage, obtained from Persian tragacanth No. 2, failed to affect the fetal development of all the 12 mother mice.

Hence, it must be assume that the embryotoxic effect (observed after intraperitoneal injection) of different tragacanth charges of Persian origin is conditioned not by a systemic, but by a direct action on the uterus.

Since some of the investigated suspensions are used also in manufacturing suspensions to be orally applied, and since tragacanth as well as guar and agar are allowed as food thickeners, the fetotoxic effect of these natural substances, as compared with gum arabic and talc, was tested by oral application also on 35 mice. The soundings (probings) were undertaken from the 11th to the 15th day of gestation daily in amounts of 0.5 ml per animal in the form of 1% and 10% suspensions or mucilages. Despite this high dosage, the proportion of fetal resorption, the average number of living fetuses,

their body lengths and weights as well as the malformation rate in the tests with Persian and Indian tragacanth, agar and gum arabic, lay within normal limits. It is only after the sounding of 10% talc suspension and of 1% guar mucilage that the proportion of fetal resorption was raised.

Thus Persian tragacanth in oral application of large amounts even, like the other natural substances tested, failed to affect the fetal development of the NMRI mice.

3. Embryotoxic Effect of Suspensions in Early Pregnancy:

Previous tests with chemical, teratogenic compounds like formamide, monomethyl formamide, 1-ethylene imino-2-oxybutene-3 and 6-mercaptopurine showed that during the early period of gestation the mice-embryos were only slightly sensitive to teratogenic noxas (OETTEL and FROHBERG, 1964/1965). Hence tests were conducted in order to determine whether the embryotoxic effect of Persian tragacanth after intraperitoneal injection during the first third of the gestation is weaker than after application between the 11th and 15th day of gravidity.

Sixteen mice were injected from the 4th to the eighth day of gestation with 0.2 ml of a 1% aqueous mucilage from Persian tragacanth intraperitoneally. For comparison purpose, mice were treated in the same way with distilled water or mucilages from different natural substances (agar, guar, gum arabic, Indian tragacanth). Like in the previous tests, the animals were caused to die on the 19th day of gestation, that is one day before the normal littering term.

In all the 31 mice treated with distilled water, agar, gum arabic or Indian tragacanth - apart from the 17.6% amount of resorption some what increased in the "agar animals" as compared with what is normal - the fetal development was not disturbed. The number of fetuses, their weight and length, the proportion of malformations and resorptions corresponded to those of the untreated mice. On the other hand, normally developed fetuses were found only in one of the 16 animals treated with Persian tragacanth. The uteri of the other 15 mother mice, when these were killed on the 19th day of gestation did not differ from those of nonpregnant animals.

Since it was to be surmized that in the case of these animals the blastocytes, through early treatment, had been killed or expelled a short time before, or at the beginning of, the implantation, in another series of tests with Persian tragacanth (No. 2) the mice were killed in the majority not a short time before littering, but already at a previous point in time. The individual results are given in Table 6.

Table 6: Embryotoxic effect of 1% Persian tragacanth mucilage (No. 2) for NMRI mice after intraperitoneal injection in the first third period of gestation
a. Day of application. b. Killing day. c. Number of mother animals
d. Total number per group e. With miscarriaged fetuses f. Implantations
g. Average per mother animal i. % refer to the total number of implantations
j. Miscarriages k. Living fetuses [a] = 2/95 fetuses were malformed (cephalic monster, cleft palate) [b] = Calculated without animals with questionable early miscarriage.

a Applikationstag p.c.	b Tötungstag p.c.	c Muttertierzahl		f Implantationen		Resorptionen		j Aborte		k Feten lebend	
		d ges.	e mit miß-geb. Feten	g ges.	Ø	g ges.	%	g ges.	%	ges.	0
		2 × 0,2 ml/Maus i.p.									
4 + 5	19	9	2	96	10,7	1	1,0	0	0	95[a]	10,6
6 + 7	12	7	0	79	11,3	2	2,5	28	35,4	49	7,0
7 + 8	12	6	0	50	8,3	23	46,0	10	20,0	17	2,8
		4 × 0,2 ml/Maus i.p.									
4 – 7	7	7	0	38	5,4	0	0	25	65,8[b]	13	1,9
		5 × 0,2 ml/Maus i.p.									
4 – 8	8	9	0	38	4,2	0	0	35	92,1[b]	3	0,3

g ges. Gesamtzahl pro Gruppe, Ø = Durchschnitt pro Muttertier, i % = bezogen auf Gesamtimplantationszahl.
[a] 2/95 Feten waren mißgebildet (Kopfmonster, Gaumenspalte).
[b] Berechnet ohne Tiere mit fraglichem Frühabort.

After the twice administered intraperitoneal injection on the 4th and 5th day of gestation of in each case 0.2 ml of the tragacanth mucilage, a does which injected in the middle of the gestation had always produced 100 resorptions and miscarriages, the fetal development was not disturbed.

The same dose, administered on the 6th and 7th day of gestation, caused vaginal bleedings in 3 of the 7 mother animals. Hence, these mice, and also those treated on the 7th and 8th day of gravidity, were caused to die not one day before littering, but already on the 12th day post coitum. At this moment, fetuses (66) still living in the uteri were found only in 6 of the 13 mice. In 4 of the 13 mothers all the fetuses had been expelled. It was possible, however, to determine the number of the expelled embryos by observing the places of implantation that were still visible - in the form of "metrial glands".

On the other hand, in 6 out of 16 mice, which had been treated from the 4th to the 8th or from the 4th to the 7th day of gestation with tragacanth mucilage and had been killed immediately, the uteri did not differ from those of nonpregnant mice. Since, however, in 2 of the 16 animals, normal embryos, corresponding in their development to the 7th or 8th day of gestation, were present in the uteri, and since in the other mice the places of implantation were clearly recognizable on the mesometrium insertion, it must be assumed that in the 6 animals without "metrial glands" the embryos were expelled already before, or at the beginning of, the embedding process.

Thus the embryotoxic effect of Persian tragacanth was weaker in the case of injections administered during early gestation than in application in the middle of the gravidity period. As is shown by the tests, the mice-embryos with increasing age become more sensitive to the embryotoxic effect of Persian tragacanth; whereas Persian tragacanth mucilage injected on the 4th and 5th day of gestation failed to disturb the embryo development, fetal death and miscarriage or resorption occurred after injection on the 6th and 7th day of gestation, and still more markedly after application on the 7th and 8th day of gravidity.

4. Comments on the Problem of the Antineoplastic Effect of Suspensions:

ROE (1959), GALBRAITH, MAYHEW and ROE (1962) as well as MAYHEW and ROE (1964) found that native Persian tragacanth, but not heated Persian tragacanth and Karaya rubber (= Indian tragacanth) inhibited the growth of mice-ascites-tumors.

Tabelle 7. *Antineoplastische Wirkung von Naturstoffen am Ehrlich-Ascites-Carcinom der Maus. 5 × 0,2 ml/Tier i.p. einer 1%igen wäßrigen Suspension (Montag bis Freitag). (Erste Injektion 2 Std nach der Tumortransplantation)*

a Substanz	b Lebensdauer in Tagen		e Ascites-menge beim Tod (g)	f Tumor-hemmung in %
	c Mittel-wert	d Absolute Streuung „range"		
Versuchsreihe I				
h Unbehandelt	11,9	8—15	9,6	—
i Aqua dest.	11,5	7—15	9,1	4,4
j Gummi arabicum 10%	8,2	6—12	4,1	57,6
k Talkum 10%	9,1	7—12	5,2	45,3
l Ind. Traganth (Nr. 2) 1%	9,0	8—15	5,0	47,5
m Pers. Traganth (Nr. 0) 1%	6,8	4— 8	1,6	83,2
Versuchsreihe II				
Unbehandelt	14,9	12—18	9,3	—
Aqua dest.	12,8	7—17	8,0	13,7
Gummi arabicum 10%	11,3	9—14	5,1	45,1
Talkum 10%	10,7	4—15	4,4	52,5
Ind. Traganth (Nr. 1) 1%	10,2	8—13	4,2	54,4
Pers. Traganth (Nr. 0) 1%	8,1	5—14	0,5	94,7
Versuchsreihe III				
Unbehandelt	11,6	9—15	6,2	10,5
q Agar (Fäden) 1%	9,1	8—13	2,0	67,7
p Carrageen 1%	11,0	8—16	5,6	10,5
r Guar 1%	9,1	8—12	2,8	55,2

Bei der Tumortransplantation wurde den Mäusen jeweils 1 ml verdünnter, frischer Ascites i.p. injiziert. Zur Verdünnung wurde Ringerlösung verwandt. Das Verdünnungsverhältnis betrug in der Versuchsreihe I 1:1, in den Versuchsreihen II und III 1:10.

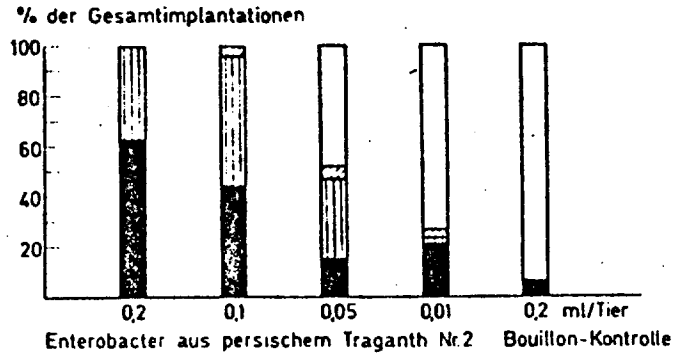


Abb. 5. Embryotoxische Wirkung kleinerer Dosen von Enterobacter-Bouillon-Sterilfiltraten für NMRI-Mäuse einmal i.p. am 12. Tag p.c. ■ Resorptionen, □ Aborte, □ Mißbildungen, □ lebende Feten

In order to obtain an accurate determination of the isolated gram negative germs, the following properties were tested regularly, or the following reactions were conducted:

Motility
Hemolysis
Gelatin liquefaction
H_2S formation
Urea dissociation (splitting)
Methyl red reaction
Voges-Proskauer reaction
Indole reaction
Koser citrate
"Multicolored Series" with the following hydrocarbons and alcohols: dextrose, lactose, saccharose, salicin, inositol, adonitol, dulcitol.

For further differenciation, in individual cases, the following culture characteristics were investigated also:

Splitting of sorbitol, xylose, arabinose, starch.
Behavior in litmus milk, nitrate reduction, detection of Phenylalanine-decarboxylase, lysine-decarboxylase, catalase, cytochrome-oxydase.

In the case of the gram positive bacilli, the following culture characteristics were used for diagnostic purpose:

Growth in broth and on agar
Motility
Hemolysis
Gelatin liquefaction
Splitting of dextrose, lactose, saccharose, xylose, mannitol
Indole reaction
Voges-Proskauer reaction.

In the case of the isolated gram positive diplococci, the following investigations were conducted:

Growth in broth and on agar
Hemolysis
Splitting of dextrose, saccharose, lactose, mannitol, maltose, salicin, sorbitol, induline, aesculine
Indole reaction.

On the basis of the undertaken investigations, the germs, isolated out of the different tragacanth specimens, were determined as follows: Results in Table 8.

Table 8: Bacteriological findings relative to different tragacanth specimens
a. Tragacanth specimen b. Yeasts c. Fungi

a. Traganth-probe	b. Bakterien		c. Hefen Pilze
	gramnegativ	grampositiv	
Pers. Traganth			
1	Enterobacter aerogenes	—	—
2	Enterobacter aerogenes	—	—
3	Enterobacter aerogenes	—	Penicillium
4	Enterobacter aerogenes	—	—
5	Enterobacter aerogenes	—	—
6	Enterobacter aerogenes Flavobact. rhenanus	Bac. circulans Bac. subtilis Diplococcus (nicht differenziert)	Mucor
7	a) Enterobacter aerogenes b) Enterobacter aerogenes	Bac. megatherium Diplococcus (nicht differenziert)	—
Ind. Traganth			
1	—	Bac. mesentericus Bac. subtilis	— —
2	—	Bac. mesentericus Bac. subtilis	—

After these investigations, enterobacter aerogenes germs were detectable in all the Persian tragacanths, and even as pure culture in specimens 1 to 5.
In the gragacanth specimen No. 6, a flavobacterium rhenanus, belonging to the family of the achromobacteraceae, was still isolated. In the tragacanth specimens

No. 6 and 7, it was possible to detect, in addition to enterobacter-aerogenes germs and the flavobacterium, gram positive aerobic sporiferous (bacteria ?) as well as gram positive diplococci, which were not more closely differentiated. In the tragacanth specimens No. 3 and 6, a penicillium or a Mucor germ was detectable; the latter was not included in the further investigations.

In contrast with the results of bacteriological investigations, in the Persian tragacanth specimens of different origins, no germs had grown in the parallel text with the two Indian tragacanths after an incubation of 24 hours. It was only after a storage of 36 to 48 hours at + 37 C, that on the nutrient agar dishes different gram positive aerobic sporiferous bacteria (Bac. subtilis, Bac. mesentericus) were detected. The only low number of bacteria in the Sterculia rubber should be attributed to the fact that the commercial product is solubilized through the autoclave (Merck Index, 8th Edition 1968).

According to the investigations conducted so far, the gram negative germs contained in the Persian tragacanth specimens might be regarded as the cause of the embryotoxic effect. Hence, tests were performed in order to determine the possibility of isolating these gram negative bacteria from the absominal cavity, after repeated intraperitoneal tragacanth injection.

For this purpose, two mice, which had been treated intraperitoneally one, 3 or 5 times with 0.2 ml of a 1% mucilage of Persian or Indian tragacanth, were caused to die nearly 8 hours after the last injection; the abdominal cavity was opened under sterile conditions, the peritoneal fluid between the partly adherent intestinal loops were removed, and inoculated on agar dishes.

It was possible to isolate the enterobacter germs, detected bacteriologically in the different Persian tragacanth specimens - although these germs were few - from the absominal cavity of the mice treated intraperitoneally 4 or 5 times with these mucilage specimens. In the test with Persian tragacanth No. 6, the gram positive diplococci were found again in the abdominal cavity of the animals treated with this tragacanth. Nevertheless, it was impossible to isolate the gram positive aerobic sporiferous bacteria, primarily detected in the Persian tragacanth specimens No. 6 and 7 or in the Indian tragacanth No. 1, from the

abdominal cavity of the mice treated repeatedly intraperitoneally in the case of these specimens.

7. Embryotoxic Effect of Products of Metabolism of Bacteria from Persian Tragacanth:

In order to exclude a bacterially induced peritonitis as cause of the embryotoxic effect of Persian tragacanth, tests were conducted in order to ascertain whether products of metabolism of the isolated germs can also exert an embryotoxic effect. For this purpose, normal nutrient broth was inoculated with pure cultures of the gram negative germs isolated from the different Persian tragacanth specimens (2 Pt loops of a 24-hour culture on 50 ml of broth) and was incubated during 48 hours at +37 C. Then these broth cultures were passed under sterile conditions through bacteria-proof filters (Seitz/Filter - Layer EKS II (EKS = sterilization layer)). After verifying the sterility by preparing smears on agar dishes, these broth cultures made sterile by filtration were injected under sterile conditions with the products of metabolism of the enterobacter strains in mice intraperitoneally - starting on the 11th day of gestation - in doses of 0.2 ml per animal (Figure 4).

Figure 4: Embryotoxic effect of broth cultures, filtrated under sterile conditions, of enterobacter strains for NMRI mice. 1 x 0.2 ml/animal intraperitoneally on the 11th day of gestation Resorptions, Miscarriages, Dead fetuses, Living fetuses, Malformations.

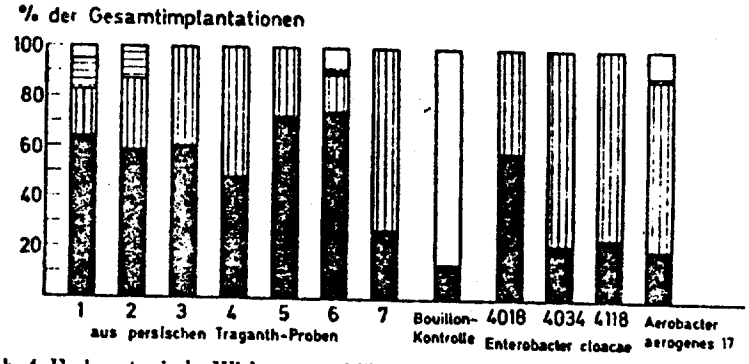


Abb. 4. Embryotoxische Wirkung steril filtrierter Bouillon-Kulturen von Enterobacter-Stämmen für NMRI-Mäuse. 1 × 0,2 ml/Tier i.p. am 11. Tag p.c. ■ Resorptionen, ▤ Aborte, ▥ tote Foten, ▭ lebende Foten, ▨ Mißbildungen

Already in the course of 12 to 15 hours after the first intraperitoneal injection, vaginal bleedings - which must be regarded as sign of miscarriage - developed in 7 of the experimental groups, which had been treated with enterobacter broth culture filtrates from the Persian tragacanth specimens No. 1 to 7, in the majority of the mice. Hence, the mice of these 7 experimental groups, which for a certain time (1 - 2 days) exhibited a ruffled skin and retracted flanks, were treated only once. Three mother animals died intercurrently.

Despite the only single intraperitoneal injection of the Persian tragacanth specimens No. 1 - 7, living fetuses were found in uteri only in 2 of the 55 mother mice treated. All the other 543 fetuses had been resorbed or had been expelled as abortions.

The 13 mice of the two other experimental groups developed no vaginal bleedings, hence they were treated from the 11th to the 15th day of gestation. In this experimental setup, the two sterile filtrates, which contained products of metabolism of the flavobacterium rhenanus and of an enterobacter aerogenes, exerted an embryotoxic effect, since about half the fetuses of the mother animals were rsorbed or expelled as miscarriage. The embryotoxic action of the products of metabolism of this flavobacterium rhenanus and of this enterobacter strain, however, was considerably weaker than that of the other enterobacter strains, which already after the single injection practically caused the death of all the fetuses. In its cultural properties also, the enterobacter germ differed from those of the other enterobacter strains.

These investigations reveal that the sterile filtrates of broth cultures, inoculated with enterobacter germs and incubated for 48 hours at +37 C, exerted an embryotoxic effect.

In order to determine whether other strains of the same genus had an embryotoxic effect, sterile filtrate of broth cultures of the aerobacter aerogenes strain 17 (Dr. F. Selenka) and of 3 enterobacter cloacae strains (Dr. B. Schmidt) were tested (The long footnote at this point merely expresses thanks to these two university lecturers).

The sterile filtrates of the enterobacter strains defined caused the death of 241 out of 253 fetuses of the 34 mother mice, after a single injection on the 11th day of gestation (See Figure 5 p. 14a).

A 1% aqueous mucilage from Persian tragacanth, after repeated subcutaneous injection, had no embryotoxic effect. Hence, a broth culture made sterile by filtration was prepared from the same gragacanth charge in the way described above. This filtrate was injected under sterile conditions subcutaneously in 18 mice from the 11th to the 15th day of gestation in doses of 0.2 ml/animal. Furthermore 5 gravid mice were treated in the corresponding way with a broth culture made sterile by filtration; this filtrate had been prepared with enterobacter cloacae 4034. The 8 control mice received individually 0.2 ml of empty broth.

The fetal development of the 23 mother animals, treated with sterile filtrates of broth culture (obtained from Persian tragacanth No. 2 and from enterobacter cloacae 4034), did not differ from the 8 control mice treated with empty broth.

These "subcutaneous tests" corroborated the assumption that the embryotoxic effect (observed after intraperitoneal injection in NMRI mice) of tragacanth contaminated with bacteria of the genus enterobacter, is due not to a systemic but to a direct action on the uterus and on its vacular system.

In the tests reported at the beginning of this study, a single intraperitoneal injection of a 1% Persian tragacanth mucilage was followed, especially on the 12th day of gestation by increased malformations and especially by cleft palates (See Table 6). Hence, tests were conducted in order to determine whether the products of metabolism of enterobacter in doses, which practically no longer killed all the fetuses or produced resorption or miscarriage, possibly caused increased malformations.

For this test, a sterile filtrate, which had been obtained from a broth incubated for more than 48 hours with enterobacter strain 2, was injected in gravid mice in doses of 0.2; 0.1; 0.05 and 0.01 ml per animal once on the 12th day of gestation.

Like in the previous tests, on the 11th day of gestation, after a single intraperitoneal injection on the 12th day of gestation of 0.2 ml/animal of the sterile filtrate of broth culture, practically all the fetuses were resorbed or were expelled as miscarriage. The two surviving fetuses of the "0.1 ml group" had uranoschism. After the injection of 0.05 ml/animal, about half of the fetuses were killed, and even after the administration of only 0.01 ml/mouse still 16 of the 75 fetuses were resorbed, and 2 were expelled as miscarriage. Four of the 79 living fetuses of these two series of tests had malformations, especially cleft palates and some malformations of the extremities with extended subcutaneous bleedings.

In order to exclude the possibility for products of metabolism of the gram positive germ found in the different tragath specimens to possess an embryotoxic action, broth cultures made sterile by filtration were prepared under the same conditions as in the case of the gram negative germs from the different gram positive aerobic sporiferous bacteria (Bac. mesentericus and subtilis) and diplococci, which had been isolated from Persian and Indian tragacanth; and these cultures were used in injecting intraperitoneally mice from the 11th to the 15th day of gestation with 0.2 ml/animal daily.

In the same way, sterile filtrates of broth cultures of different other germs, stemming from the collection of the Industrial Hygiene Pharmacological Institute, such as E. coli I, Staph. aureus SG 511, Bac. subtilis and Bac. mesentericus, were tested.

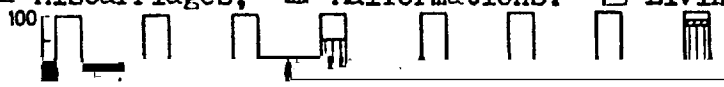
Neither the sterile filtrates of broth cultures of the gram positive germs isolated from Persian and Indian tragacanth, nor the concomitantly tested control filtrates of E. coli, Staph. aureaus SG 511, Bac. subtilis and Bac. mesentericus possessed an embryotoxic action on the 103 gravid NMRI mice used for these tests.

8. Effect of the Use of the Autoclave on the Embryotoxic Action of Sterile Filtrates of Enterobacter Broth Cultures:

In order to find out whether through the use of the autoclave the embryotoxic action of products of metabolism contained in the sterile filtrates of broth cultures of enterobacter strains is decreased or eliminated, sterile filtrates of broth cultures of the strains Aerobacter aerogenes strain 17, Enterobacter cloacae 4034, Enterobacter strain from Persian tragacanth No. 2 and Enterobacter strain from Persian gragacanth No. 6 were subjected to a single treatment for 15 minutes at 120 C in the autoclave. These sterile filtrates of broth culture, thus treated, were injected once daily intraperitoneally in gravid mice once 0.2 ml/ animal on the 11th day of gestation or from the 11th to the 15th day of gestation in amounts of 0.2 ml/ animal (See Figure 6).

Figure 6: Embryotoxic effect of broth cultures (obtained by sterile filtration) from Enterobacter strains after the use of the autoclave for NMRI mice.

Resorptions. Miscarriages, Malformations. Living fetuses.



a single injection of 1% mucilage from Persian tragacanth. Hence, the peritoneal stimulation produced by this tragacanth after intraperitoneal injection should not be the cause of its embryotoxic action.

In contrast with the intraperitoneal tests, Persian tragacanth mucilage in oral application even after repeated administration of large amounts, affects the fetal development of mice just as little as the other tested natural substances. Hence - as far as a posteriori conclusions can be drawn from animal tests and applied to human beings - there should be no hesitation, from the embryotoxic effect standpoint, to continue the utilization of Persian tragacanth, beside other natural substances, as an addition to food.

A cytotoxic effect, described by ROE (1959), GALBRAITH, MAYHEW and ROE (1962) as well as MAYHEW and ROE (1964) in the case of Persian tragacanth on the basis of investigations with some mice-ascites-tumors, must be excluded, according to the investigations presented here, as cause of the observed embryotoxic effect of pure tragacanth, since the Persian tragacangh specimens, that we tested, like the other natural substances, possess on the Ehrlich ascites carcinoma of the mouse no significant antineoplastic effect after intraperitoneal injection.

According to our investigations, it is rather bacteria or their products of metabolism that must be made responsible for the embryotoxic effect of the Persian tragacanth charges tested. This is shown already by the decrease of the embryotoxic action of aqueous mucilage from Persian tragacanth after sterilization, and especially by the presence of gram negative coccoid rods of the genus Enterobacter in all the 7 investigated Persian tragacanth specimens, which are not found in the two Indian tragacanth specimens. The considerable lack of bacteria in Sterculia-rubber should be attributed to the solubilization of this commercial product through the autoclave (Merck Index, 1968). The products of metabolism of the individual isolated Enterobacter strains, injected intraperitoneally in the form of sterile filtrates of broth cultures in gravid animals, exert a higher embryotoxic effect than the original tragacanth mucilage. The products of metabolism of bacteria of these Enterobacter strains should represent thus the real active form. The control tests, conducted with defined Enterobacter strains, show that the embryotoxic effect is specific for the bacteria products of metabolism of the isolated bacteria genus (Enterobacter).

Discussion:

Distilled water, Ringer's solution, the 1% mucilages of gum arabic, carrageen or guar, used in manufacturing suspensions or emulsions, and even 1% aqueous talc suspensions do not influence the fetal development of NMRI mice after a single intraperitoneal injection, or after several, in the sensitive phase of gestation. Under the same experimental conditions, however, once mother mice have been treated with 1% aqueous mucilages from 8 different Persian tragacanth specimens, the death and subsequent resorption or expulsion of all the fetuses occur. On the other hand, mucilage from 2 Indian tragacanth specimens exerts no effect, like the other natural substances tested, on the fetal development.

The mice-fetuses most easily injured are those involved in the treatment administered in the middle of the gestation period (11th and 12th day of gravidity). In the stage of advanced organogenesis, the fetuses become increasingly more resistant to the embryotoxic effect of Persian tragacanth. Thus the sensitivity of mice-embryos to pure tragacanth, during their development, is just as different as to known chemical or physical teratogenic noxa (WILSON, 1965; RUGH and GRUPP, 1959 as well as OETTEL and FROHBERG, 1965, 1965).

In all the mother mice, treated with a 1% aqueous Persian tragacanth mucilage intraperitoneally, dissection revealed inflammatory changes in the abdominal cavity. Hence at first it was assumed that the peritonitis, produced by the intraperitoneal injection, was the cause of the fetal injury, especially other natural substances, which do not affect the fetal development, like the investigated Indian tragacanth-, agar, or carrageen specimens which even after repeated intraperitoneal injection in the form of 1% aqueous mucilages do not exert a stimulating effect. There is an objection, however, against peritonitis as cause of the embryotoxic effect of Persian tragacanth: 1% aqueous gum arabic and especially talc suspensions after repeated intraperitoneal injection in mice cause stronger inflammatory stimulations in the abdominal cavity than Persian tragacanth, although these suspensions have just as little effect on the fetal development as Ringer's solution or distilled water. Even through five time intraperitoneal injection of a 10% aqueous gum arabic or talc suspension, the fetal development , despite most severe peritonitic inflammations and massive adherences of the ingesta in the mother animals is less disturbed than through

The gram positive aerobic sporiferous bacteria and diplococci, isolated from Persian tragacanth, generate no products of metabolism with embryotoxic action. In the same way behave also the gram positive germs, which were isolated from the Indian tragacanth specimens, or had been borrowed from the collection of our Institute. The broth culture, filtrated under sterile conditions, of an E. coli-strain used for comparison did not have an embryotoxic effect either.

Hence, according to the investigations, the gramnegative germs, isolated from the different Persian tragacanth specimens, of the genus Enterobacter or their products of metabolism are the cause of the embryotoxic effect of Persian tragacant after intraperitoneal injection for NMRI mice. Nothing precise can be stated about the nature of these products of metabolism of bacteria. Since, however, the used of the autoclave for 15 minutes at 120 C only weakens, but does not eliminates, the ambryotoxic effect of sterile filtrates of broth cultures of Enterobacter, the effective substance must consist at least of a thermolabile and of a thermostable constituents. Whether the thermolabile constituents involves albumins, and the thermostable part possible polysaccharides - like this is known from endotoxins of gram negative germs, must be clarified through further investigations.

Our investigations with decreasing doses of a sterile filtrate of broth culture of enterobacter on the 12th day of gestation show that the number of the dead fetuses decreases in proportion to the dose injected; but that malformations are not increased. Hence it can be concluded that the resorptions and miscarriages, observed in our tests, very probably are no signs of a pure teratogenic action of the products of metabolism of the bacteria.

It is possible that the fetal death is due to massive placenta bleedings, caused by the products of bacteria metabolism of the Enterobacter strains. This might shown by the observation that in some mice, killed immediatly after vaginal bleeding was clinically found, fresh bleedings were detected on the surfaces of the placenta without any fetal residues (state after abortion) and in those with attached fetal sack in which the fetuses in the process of resorption lay. Placenta bleedings, connected with the death of the fetuses, were observed also by THIERSCH (1960) in pregnant rats after injection of lipopolysaccharides, obtained from gram negative bacteria. The cause of the placenta bleedings

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could be a disturbance of the blood coagulation, since for dogs, intravenous injections of tragacanth mucilage are deadly on account of the effect exerted on the blood coagulation (WALTON and others, 1959). Such a mechanism could also be implied by the increase of the vascular permeability and lengthening of the time of coagulation found as a result of lipopolysaccharide action, by WESTPHAL and Collaborators (1955).

After repeated subcutaneous injection, a tragacanth mucilage, contaminated with bacteria of the genus Enterobacter, as well as the sterile filtrates of broth cultures manufactured from this tragacanth charge and Enterobacter cloacae 4034, did not influence the fetal development of the NMRI mice. Hence it must be assumed that the embryotoxic effect, observed after intraperitoneal injection in NMRI mice, of different Persian tragacanth charges, which were contaminated with bacteria of the genus enterobacter, and of sterile filtrate of broth cultures of Enterobacter, is not due to a systemic effect, as for instance a general disturbance of the blood coagulation, but to a direct action on the uterus and its vascular system.

For the practice, it results from the investigations presented in this study that:

1. Suspensions and emulsions, which can be contaminated by bacteria of the genus Enterobacter, should at much as possible not be used in testing the possible teratogenic effect of water insoluble substances on the mouse by means of intraperitoneal injection, and

2. Substances, which cause an increasing of the vascular permeability, or possess considerable vasodilatatory or vasoconstrictory effect, and must be applied parenterally, should not be injected intraperitoneally when testing the teratogenic effect, in order to prevent a fetal injury caused by a local perfusion disturbance of the uterus.

APPLIED MICROBIOLOGY, Jan., 1967, p. 31-34
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Vol. 15, No. 1
Printed in U.S.A.

Antibiotic Activity in the Presence of Agar

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Received for publication 20 June 1966

ABSTRACT

Agar has been shown to interfere with the activity of some antibiotics against *Staphylococcus aureus*. This interference has been observed as an increase in the minimal inhibitory concentration and in the diameter of the zone of inhibition. Purifying the agar with water extractions substantially reduced this adverse effect.

Previous studies in this laboratory have shown that the presence of agar in a test medium can seriously interfere with the inhibitory activities of antimicrobial agents (13, 24, 25). In an effort to overcome this interference, agar was extracted with water prior to use in the medium. The washed agar medium was found to be less antagonistic for these inhibitors, and the minimal inhibitory concentration in this medium often approached those in nutrient broth.

It was felt that this antagonistic effect might also extend to the antibiotics. If this were true, it could be an important factor in both the detection and use of these agents. It is well known that in the search for new antibiotic-producing organisms the initial screening tests are routinely performed in an agar medium, and quite often the antibacterial spectrum of a new antibiotic is determined by the agar plate dilution technique (2, 18). In addition, a semisolid medium is routinely used in sensitivity tests to establish the best antibiotic for use in clinical therapy.

This investigation was therefore carried out to determine whether the presence of agar in the test medium had any adverse effect upon the activities of antibiotics, and whether this antagonism could be reduced by the use of washed agar.

MATERIALS AND METHODS

The culture of *Staphylococcus aureus* employed in previous studies (13, 24, 25) was used throughout this investigation. The organisms were stored on nutrient agar at 4 C and were transferred every 4 days. The inoculum was prepared by growing the organisms for 24 hr at 37 C on nutrient agar and washing them from the agar in sterile isotonic saline. A Klett-Summerson photoelectric colorimeter with a blue filter was used to obtain a uniform turbidity of 100 Klett units. After a 10 fold dilution, this suspension contained approximately 2.4×10^7 cells per milliliter.

Concentrated aqueous solutions of the antibiotics were filter-sterilized through an ultrafine Morton fritted-glass filter, and were aseptically diluted to the desired concentration in sterile, distilled water.

The washed agar cubes (3% Difco agar) were prepared in the same manner as described in previous studies (13, 24). The cubes were melted under flowing steam in the autoclave prior to use in the test medium; 3% aqueous solutions of unwashed Difco agar were melted in a similar manner.

Double-strength nutrient broth was prepared, and was divided into three portions. One portion was diluted to single strength with melted, washed agar; the second portion was diluted to single strength with melted, unwashed agar; and the third portion was diluted to single strength with deionized, distilled water. In each of the semisolid media, the final agar concentration was 1.5%.

A 5-ml amount of each medium was pipetted into a series of screw-cap tubes and was autoclaved for 15 min at 121 C. The tubes were then placed in a water bath and were brought to a temperature of 46 C. The tubes were removed individually from the water bath, and 0.1 ml of the antibiotic solution was added aseptically. The tube was then vigorously shaken on a Vortex Jr. mixer. A 0.1-ml amount of the standardized cell suspension was then added, and the tube was again shaken. When the agar had solidified, the tubes were incubated at 37 C for 48 hr. At the end of this period, the tubes were examined under 10× magnification for growth.

The approximate inhibitory concentration for the organism was first determined by a screening test in which concentrations of 0.01, 0.1, 1.0, 10, 100, and 1,000 ppm were used. Once the inhibitory range was determined, it was divided into 10 increments. For example, if the range was 0.1 to 1.0, the increments in this range were 0.1 ppm. Two additional experiments were performed with the use of the smaller increments. All data from these experiments are given in parts per million (ppm) of antibiotic (w/v).

The media for the agar plates were prepared in the same manner as for the tubes. They were autoclaved, cooled to 45 C, and seeded with the standardized suspension of *S. aureus*, and 10 ml was pipetted into sterile petri plates. Antibiotic sensitivity discs (Difco) of intermediate concentration were placed on the

solidified agar, and the plates were refrigerated at 4 C for 2 hr. They were then incubated at 37 C for 48 hr and were examined.

RESULTS

In the control tubes containing no antibiotic, heavy growth occurred at the surface of the agar and slightly below the surface. In subinhibitory concentrations of antibiotics, the organism consistently produced individual colonies on the surface and slightly below.

When the sensitivity of *S. aureus* to the tetracyclines obtained in nutrient broth was compared with that found in nutrient broth solidified with unwashed Difco agar, it was noted that the minimal inhibitory concentrations (MIC) were only slightly higher in the agar medium (Table 1). A comparison of the washed and unwashed agar media reveals that use of the washed agar medium reduced the MIC by one-half. This difference is statistically significant.

The antibiotics shown in Table 2 were not significantly affected by the presence of unwashed or washed agar in the medium.

The activities of the antibiotics presented in Table 3 were markedly affected by the presence of unwashed agar in the test medium. There was a 55-fold increase in the MIC of polymyxin in the semisolid medium, a 30-fold increase for neomycin, a 6-fold increase for kanamycin, and approximately a 3-fold increase for streptomycin. Although the MIC for these compounds was higher in the washed agar medium than in nutrient broth, the major portion of the total antagonism was removed from the agar by washing.

TABLE 1. *Effect of agar on the inhibitory activity of the tetracyclines against Staphylococcus aureus*

Compound	Minimal inhibitory concn (ppm)		
	Nutrient broth	Unwashed agar	Washed agar
Aureomycin hydrochloride	0.15	0.27	0.11
Terramycin hydrochloride	0.46	0.68	0.34
Declomycin hydrochloride	0.07	0.13	0.06

[a] Average of four determinations.

TABLE 2. *Antibiotics that are not significantly affected by the presence of agar*

Compound	Minimal inhibitory concn (ppm)[a]		
	Nutrient broth	Unwashed agar	Washed agar
Vancomycin	0.5	0.7	0.5
Novobiocin sodium	0.9	1.0	0.8
Penicillin G sodium	0.1	0.1	0.1

[a] Average of four determinations.

TABLE 3. *Antibiotics that are adversely affected by the presence of agar*

Compound	Minimal inhibitory concn (ppm)[a]		
	Nutrient broth	Unwashed agar	Washed agar
Polymyxin	4.0	220.0	9.0
Neomycin	0.1	3.0	0.45
Kanamycin sulfate	0.5	3.0	0.6
Streptomycin sulfate	2.25	6.25	4.0

[a] Average of four determinations.

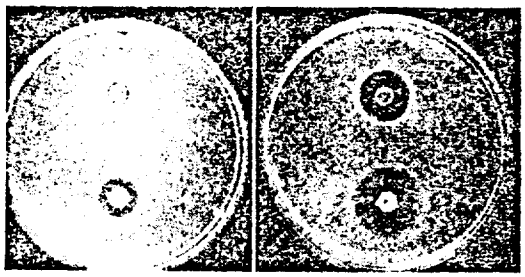

FIG. 1. *Zones of inhibition on unwashed agar medium (left) and washed agar medium (right). Upper disc contains neomycin, lower disc contains dihydrostreptomycin.*

Figure 1 illustrates the effect of washed Difco agar on the zones of inhibition produced by antibiotic discs of neomycin and dihydrostreptomycin. The increase in size of the zone of inhibition produced by neomycin on the washed agar is especially noticeable. Similar experiments with the tetracyclines showed only a slight increase in the size of the zone on washed agar.

DISCUSSION

Our results show that the presence of agar in the test medium may markedly affect the activity of an antibiotic against *S. aureus*. This is to be expected because agar is a chemically reactive substance. According to Araki (1), agar is a complex substance composed of at least two polysaccharides: agarose, which contains little sulfate and no acid groups, and agaropectin, which is sulfated and contains acid residues.

Agar carries an overall negative charge, probably due to the acid and sulfate groups. Because of the presence of these charged groups, agar may

react with protein molecules (3, 32) and basic groups of other molecules such as polymyxin and neomycin (9).

Divalent metallic cations have been shown to reduce the antibiotic effects of polymyxin, neomycin, streptomycin, novobiocin, and particularly the tetracyclines (7, 20, 30, 31, 33). It has been demonstrated that calcium, magnesium, and other metallic cations are often bound to agar (1). Washing the agar indicates that substantial quantities of these ions are removed.

Traces of growth factors and stimulatory factors have also been found in agar, including thiamine, β-alanine, biotin, niacin, and *p*-aminobenzoic acid (6, 21, 22). These factors could alter an organism's sensitivity to an antibiotic in a variety of ways.

This investigation indicates that two important problems are currently being overlooked in many laboratories. First, antibiotics are routinely detected and screened in an agar medium. It is possible that many valuable antibiotics are overlooked or discarded because their effects have been suppressed by the presence of agar. Second, clinical sensitivity tests are usually carried out by use of a medium with agar and sensitivity discs. Eisenberg, Weiss, and Flippin (8) noted that, although 54 strains of *Candida albicans* were sensitive to nystatin in broth, only 11% of these were sensitive to the same concentration of this antibiotic on plates. In only 18.5% of the tests were the results of the plate and tube dilution sensitivity tests in aggreement. Vancomycin, the tetracyclines, streptomycin, nystatin, dihydrostreptomycin, and furadantin have also been shown to be affected by agar (10, 16, 17, 29). In some instances, bacteria that appear resistant to antibiotics by evaluation with sensitivity discs may be rapidly destroyed by the antibiotic in vivo (27).

To eliminate these antibiotic-testing problems, an improved solidifying agent is imperative. The technique of washing agar prior to use has been used in the fields of biochemical genetics (22), immunology (11), and virology (26, 28). This results in the extraction of biologically and chemically active factors (5, 12, 19, 23). Our results indicate that a washed agar medium is superior to an unwashed agar medium for testing antibiotic sensitivities.

Washing alone, however, is not sufficient to make agar an inert component of the test system. Agar still contains acidic groups which are capable of binding positively charged atoms or molecules (4). Several reports (3, 14, 15) indicate that a gel-forming, neutral polysaccharide containing very small quantities of SO_4^{--} can be obtained from agar. It is quite possible that its reduced chemical reactivity will render it more suitable for use in testing antimicrobial agents.

ACKNOWLEDGMENTS

We are grateful to American Cyanamid Co., Wayne, N.J., Bristol Laboratories, New York, N.Y., Eli Lilly & Co., Indianapolis, Ind., Merck & Co., Inc., Rahway, N.J., and Chas. Pfizer & Co., Inc., Brooklyn, N.Y., for kindly supplying the antibiotics used in this investigation.

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J. Nutrition 63, 1957

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GROWTH OF RABBITS ON PURIFIED DIETS [1]

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(Received for publication February 21, 1957)

The growth of rabbits fed casein-sucrose purified diets is usually less than optimum, and certain natural feedstuffs must be added for good gains. Hogan and Hamilton ('42) reported that rabbits grew normally on casein diets supplemented with yeast or liver extract. Kunkel, Simpson, Pearson, Oleese, and Schweigert ('48) also noted the beneficial effect of liver extract for rabbit growth and maintenance of the fur. Wooley ('54) reported that leafy material, such as kale, added to the casein diet greatly improved growth. Good growth was also obtained by readjusting the potassium level of the diet and increasing the casein level to 30%. The beneficial effect of soybean meal will be reported in the present paper. The rabbit has a high potassium requirement (Hove and Herndon, '55) and perhaps a high sodium and calcium requirement (Wooley and Mickelsen, '54); since little is known about the quantitative requirements of other nutrients it is hard to assess the reasons for the growth stimulation brought about by certain feed ingredients.

EXPERIMENTAL

The compositions of rabbit diets R43 and R14E are given in table 1. Variations in the level of protein source or of

[1] Supported in part by a grant-in-aid from the National Institute of Neurological Diseases and Blindness (Project B-430), and published with the approval of the Director of the Alabama Agricultural Experimental Station. Appreciation is expressed to Merck and Company, Lederle Laboratories, and A. E. Staley Manufacturing Company for vitamins used in these studies.

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supplements were compensated by adjustments in the carbohydrate level. Commercial, solvent-process soybean meal that was further subjected to a one- or 2-day continuous methanol extraction was used throughout ($N \times 6.25 = 51.3\%$ protein). The casein was also methanol-extracted.

Rabbits of the New Zealand-white or the California-white strains were placed on the experimental diets at 4 weeks of

TABLE 1

Composition of diets

	R-43	R-14E
	%	%
Casein [1]	20	0
Soybean meal [1]	0	40
Sucrose	51	32
Cellulose [2]	10	10
Salt mixture no. 5 [3]	5	5
Potassium bicarbonate	1	0
Lard	6	6
Cod liver oil	2	2
Vitamin premix [4]	5	5

[1] Continuous one- or two-day methanol extracted.

[2] "Non-nutritive fiber" of General Biochemicals, Inc.

[3] W. D. Salmon, J. Nutrition, *33:* 155 (1947).

[4] Contributed per gram diet: thiamine, riboflavin, pyridoxine, 3 μg each; calcium pantothenate, 17 μg; choline chloride, 2,000 μg; i-inositol, 200 μg; niacin, 30 μg; methyl-1,4-naphthoquinone, 0.3 μg. (Vitamin B_{12}, 30 μg/kg, and folacin, 2 mg/kg, were added to some of the diets; exps. II, III and IV.) *dl*-α-Tocopherol acetate was given as 10 mg per week, orally.

age and at body weights usually of 400 to 500 gm. The animals were housed individually on half-inch mesh screens in an air-conditioned room. Water and feed were always available to the animals.

RESULTS

The normal rate of weight gain of young rabbits fed a ground, commercial rabbit feed [4] with 18% protein was about 35 gm/day (table 2). The simplified diet containing casein as the protein source, as used in the series I experiments, did

[4] Purina Rabbit Pellets.

not produce growth equivalent to that of the commercial feed even when the casein level was raised to 50%. The data in table 2 indicate that this high level of casein was superior to lower levels. However, the rate of gain was not maintained past 6 to 7 weeks on this diet. About one-third of the animals fed the casein diets showed marked loss of fur, regardless of

TABLE 2

Rabbit growth on purified diets

(Initial body weights 400 to 600 gm; time on diets usually 32 days, except for amino acid studies which were run 14 days)

EXPERIMENT SERIES NO.	LEVELS OF PROTEIN SOURCE AND ADDITIONS	NO. OF RABBITS	RATE OF GAIN
	%		*gm/day ± S.E.*
I	Casein, 10	4	4.8 ± 0.7
	Casein, 20	10	10.4 ± 3.2
	Casein, 33	4	12.4 ± 1.9
	Casein, 50	4	23.4 ± 2.2
	Soybean meal, 40	5	27.2 ± 2.7
	Casein, 20 + soybean meal, 15	5	26.6 ± 2.0
II	Casein, 20	12	14.2 ± 2.1
	Casein, 33	10	15.7 ± 2.5
	Soybean meal, 40	8	32.2 ± 2.3
	Casein, 20 + soybean meal, 25	6	35.3 ± 2.2
	Casein, 20 + water-insol. res. soybean meal, 25	2	31.5
	Casein, 20 + trypsin-digest of soybean meal, 16	5	19.1 ± 1.0
	Casein, 20 + ash of soybean meal, 4	4	15.6 ± 1.9
	Casein, 20 + dried brewers' yeast, 25	2	36.2
	Casein, 20 + arginine, 0.8, glycine, 0.8, methionine, 0.5, tryptophan, 0.1	4	23.6 ± 1.7
	Casein, 20 + arginine, methionine, tryptophan	2	18.0
	Casein, 20 + arginine, tryptophan	2	16.2
	Casein, 20 + glycine	2	12.0
III	Casein, 18	4	4.2 ± 1.2
	Enzymatic digest of casein, 18	4	15.3 ± 0.8
	Digest of casein, 18 + arginine, glycine, methionine	4	20.1 ± 1.9
	Soybean meal, 36	4	30.5 ± 3.5
IV	Casein-casein digest 1:1, 18	4	16.9 ± 2.4
	Casein-casein digest, 18 + arginine, glycine, methionine, tryptophan	7	20.8 ± 1.1
	Commercial rabbit feed (ground)	4	34.3 ± 1.5

casein level. Simplified diets containing soybean meal as the sole protein source, or added to the 20% casein diet, permitted growth in rabbits at a rate only slightly less than that of the commercial feed.

The growth-promoting properties of soybean meal could not be removed by water extraction (experiment series II, table 2). The water-soluble extract of the soy-bean meal was dried and added to the casein diet at a 3% level, but it did not improve growth (these data not shown). A water suspension of finely ground soybean meal was digested for 6 days with 1% trypsin (1–300). The filtrate was dried and fed at a 16% level; this gave a significant but not maximum growth stimulus. Dried brewers' yeast was as effective as soybean meal. The ash of soybean meal did not stimulate growth.

The additions of the amino acids arginine, glycine, methionine, and tryptophan to the 20% casein diet resulted in a significant growth increase. However, amino acid supplementation produced erratic results, and in a rather large number of other trials no benefit at all was apparent. This contrasts with the consistent and dramatic response to soybean meal.

In some experiments (as in III, table 2) an 18% casein level, with only 3% of cellulose, was used. The substitution of an enzymatic digest of casein[5] for the extracted casein improved growth significantly. The addition of the amino acids arginine, glycine, and methionine to this diet resulted in a further improvement of growth, but the growth rate did not equal that produced by the soybean meal diet. Reported growth factors, such as thioctic acid (10 mg/kg diet) and orotic acid (100 mg/kg diet), were included in the vitamin mixture used for experiment III; no benefit from these factors could be discerned.

The dried ceca (with contents) of rabbits fed the 20% casein diet were significantly heavier than those of the animals fed the soybean meal diet. Expressed as percentage of body weight, these values for 6 animals/group were 2.71 ± 0.15

[5] Nutritional Biochemicals, Inc., Chagrin Falls, Ohio.

and 1.73 ± 0.30, respectively. This may indicate a type of intestinal stasis with slower passage of ingesta through the tract.

The effect of the type of carbohydrate in the diet on the rate of body weight gain of rabbits is shown in table 3. All diets contained at least 5% sucrose, since this was the carrier for the vitamins. When corn starch was substituted for sucrose a slight but statistically insignificant improvement in growth resulted, regardless of whether the protein source was

TABLE 3

Effect of type of carbohydrate on rabbit growth

(4 rabbits per group with average initial weights of 350 to 410 gm; 40 days on diets)

PROTEIN SOURCE	CARBOHYDRATES ADDED TO DIET	RATE OF WEIGHT GAIN
	%	*gm/day ± S.E.*
20% Casein	Sucrose 51, cellulose 10	11.5 ± 0.93
	Cornstarch 51, cellulose 10	15.1 ± 1.88
	Cornstarch 61	13.7 ± 0.90
	Sucrose 31, cellulose 10, gum arabic 20	16.7 ± 1.28
	Sucrose 31, cellulose 10, agar 20	21.8[1] ± 1.33
	Sucrose 31, cellulose 10, triacetin 20	5.9
40% Soybean meal	Sucrose 32, cellulose 10	21.7 ± 1.88
	Cornstarch 32, cellulose 10	26.4 ± 1.44
	Cornstarch 42	25.2 ± 2.01

[1] Twenty-eight-day period.

casein or soybean meal. Omission of cellulose from either diet did not interfere with the growth rate. The addition of either gum arabic or agar, at a 20% level, resulted in significant improvement in growth rate in rabbits fed the casein diet. This is similar to the observation of Booth, Elvehjem, and Hart ('49) with guinea pigs. Triacetin-containing diets depressed rabbit growth.

The total carbohydrate of soybean is composed, according to Markley and Goss ('44), of about 4 to 6% each of cellulose, sucrose, stachyose, galactan and araban; only slight traces of starch or hemicelluloses are present. The inclusion of 25%

soybean meal in a 20% casein diet more than doubled the growth rate. This amount of soybean meal contributed about 10% of carbohydrate, 13% of protein, and 2% of ash. Since most of the carbohydrate consisted of substances known not to influence rabbit growth, such as sucrose, cellulose and stachyose, it is doubtful that the carbohydrate is a significant factor in the stimulation of growth. However, the galactans and arabans of soybean may have an effect on rabbit growth similar to that of gum arabic or agar.

DISCUSSION

The protein of soybean meal contains considerably more arginine and glycine than does casein. Supplements of these amino acids along with some methionine and tryptophan to the casein diet produced results that were erratic but positive enough to indicate that a partial deficiency of these amino acids contributed to the relatively poor growth on casein diets. Involvement of amino acids was further indicated by the better growth obtained when an enzymatic digest of casein replaced casein.

However, the erratic response would indicate that some nutrients other than the amino acids are limiting in the casein diet and are contributed by the soybean meal or yeast supplement. Almost nothing is known of the quantitative requirements of the rabbit for minerals or vitamins except for potassium (Hove and Herndon, '55); choline (Hove and others '55; pyridoxine (Hove and Herndon, '57) and niacin (Wooley, '47). Perhaps certain minerals or vitamins are supplied to the diet in insufficient or imbalanced amounts by the mineral and vitamin mixtures which, after all, are patterned upon the requirements for the growth of rats.

SUMMARY

For growth of rabbits fed a simple purified diet, defatted soybean meal was superior to casein as a protein source. With 20% casein the rate of growth was about 15 gm/day. Soybean meal as the sole source of protein at an equivalent level

permitted growth of about 30 gm/day. This compared favorably with growth of 35 gm/day for rabbits on a commercial feed. A supplement of arginine, glycine and tryptophan improved growth of rabbits fed the casein diet, and accounted for a part of the soybean meal effect. Other deficiencies in casein, as yet unidentified, were corrected by soybean meal or by dried brewers' yeast.

The type of carbohydrate in the diet had a minor effect on rabbit growth. Sucrose, starch, gum arabic, agar, cellulose and triacetin were compared. Gum arabic and agar influenced the growth rates most favorably.

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Antibiotics + Chemotherapy, 10: 409-13. 1960

Effect of Agar on the Inhibition of *Micrococcus pyogenes* var. *aureus* by Chlortetracycline and Other Antibiotics

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The use of agar as a solidifying agent in bacteriological media is a well-known and accepted procedure, and, usually, little thought is given to any influence it may exert *per se* on microorganisms. However, there have been reports in the literature of its role in reversing the action of antibiotics,[1] surface active agents,[2] and disinfectants,[3] whereas it had no effect on the antiseptic properties of mercury compounds.[4] Ley and Mueller[5] reported the presence in agar of an inhibitor for *Neisseria gonorrhoeae*. Agar hydrolysed with 0.1 per cent sulfuric acid was found to inhibit completely *Staphylococcus aureus*.[6]

Iyer and Iyer[7] observed that whereas a concentration of 10 μg./ml. chlortetracycline prevented the growth of *Micrococcus pyogenes* var. *aureus* 209P in nutrient agar, even on prolonged incubation, at this concentration, the antibiotic in nutrient broth failed to prevent the development of visible turbidity in 48 hours. The present investigation was undertaken to study the influence of agar on the inhibition by chlortetracycline of the test organism.

MATERIALS AND METHODS

Methods were generally similar to those detailed earlier.[7] Unless otherwise stated, B.D.H. agar (British Drug House Ltd., London), at a concentration of 3 per cent (weight/volume), was used throughout the study. Agar or the carbohydrates used were separately sterilized in 100 ml. Erlenmeyer flasks, and 20 ml. sterile nutrient broth was added to them. Chlortetracycline was always added to give a concentration of 10 μg./ml. of medium. The inoculum was always a 24 hour broth culture, such that the medium contained 10^5 to 10^6 cells/ml. After inoculation and incubation of the flasks at 37 C. for 24 hours, counts for the number of survivors in each experiment were made on nutrient agar plates. Extensions of these general procedures are described in the relevant experiments.

EXPERIMENTAL RESULTS

The ability of the organism to grow out in nutrient broth containing chlortetracycline and its inability to do so in nutrient agar at the same concentration of antibiotic was confirmed. The inoculum was harvested and added to give 10^7 and 10^8 cells/ml. While with both sizes of inoculum visible turbidity was found to develop in the liquid medium, no colonies developed on the agarified medium, even on prolonged incubation for five days.

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Received for Publication: November 10, 1959

TABLE I

Augmentation of the Chlortetracycline Inhibition of M. pyogenes *var.* aureus *209P by Autoclaved Agar*

Cells inoculated per ml. of each medium	Survivors per ml. of medium after 24 hours at 37 C.					
	Broth	Broth + washed agar	Broth + washed agar + chlortetracycline	Broth + unwashed agar	Broth + unwashed agar + chlortetracycline	Broth + chlortetracycline
5.0×10^5	3.22×10^8	1.11×10^5	2.65×10^5	1.69×10^7	1.13×10^5	2.99×10^5

Inhibitory Effect of Autoclaved Agar and Augmentation of Inhibition by Chlortetracycline. The purpose of these experiments was to determine whether autoclaved agar *per se* was inhibitory to the test organism. Both washed[8] and unwashed agar were used. The results are presented in table I. Autoclaved unwashed agar was found to inhibit slightly the growth of the test organism. This effect was more marked with unwashed than with washed agar. Washed agar together with chlortetracycline was no more inhibitory than the antibiotic itself. Unwashed agar, on the other hand, augmented chlortetracycline inhibition, which was 2.6 times greater than that observed with the antibiotic *per se.*

Growth of M. pyogenes *var.* aureus *in Chlortetracycline-Containing Nutrient Broth in Presence and Absence of Autoclaved Agar.* The number of cells of the test organism surviving in medicated nutrient broth in the presence and absence of autoclaved agar was counted in several experiments, the results of which are presented in table II. It will be observed that in the presence of agar the viable count was significantly lower than in its absence.

Effects of Varying Concentrations of Agar. Autoclaved pyridine extracts of agar have been reported[9] to stimulate the growth of *Nitzschia linearis* in low concentrations and inhibit it at higher concentrations. To check whether varying the concentrations of agar altered the augmentation in chlortetracycline inhibition observed at 3 per cent, flasks containing agar in the range of 0.1 to 10 per cent were set up as before and the inhibition compared with that obtained in an agar-free control flask. As may be seen from table III, with increasing agar concentrations

TABLE II

Survival of M. pyogenes *var.* aureus *209P in Nutrient Broth Containing 10 μg./ml. Chlortetracycline in Presence and Absence of Autoclaved Agar*

Experiment no.	Count per ml. in absence of agar		Count per ml. in presence of agar	
	0 hours	24 hours	0 hours	24 hours
1	7.4×10^6	1.6×10^6	7.4×10^6	9.4×10^3
2	8.8×10^6	3.9×10^5	1.7×10^6	4.1×10^4
3	7.7×10^5	4.1×10^5	6.3×10^5	9.7×10^4
4	2.9×10^5	2.6×10^5	2.9×10^5	2.3×10^4
5	5.7×10^5	8.0×10^5	5.7×10^5	2.1×10^5
6	5.0×10^5	2.9×10^5	5.0×10^5	1.1×10^6

TABLE III

Effect of Varying Agar Concentrations on Chlortetracycline Inhibition of M. pyogenes *var.* aureus *209P in Nutrient Broth*

Cells inoculated per ml. of each medium	Survivors per ml. of medium after 24 hours at 37 C.				
	Per cent agar				
	0.0	2.0	3.0	4.0	5.0
5.7×10^5	8.0×10^5	5.8×10^5	2.8×10^5	2.1×10^5	9.3×10^4
	6.0	7.0	8.0	9.0	10.0
5.7×10^5	2.7×10^4	3.7×10^3	4.5×10^3	4.3×10^3	2.5×10^3

a general increase in inhibition was noted. Increased inhibition was observed only with concentrations of agar greater than 2 per cent, the lower concentrations having no effect.

Effect of Some Carbohydrates as Compared to That of Agar. Glucose, sucrose, dextrin starch, and agar were autoclaved and tested at a concentration of 3 per cent. It may be noted from the results presented in table IV that the increased inhibition due to agar was not observed with any of the other compounds tested, some of which even slightly reversed the inhibition. The effect of agar appears to be specific.

Effect of Agar on Inhibition by Other Antibiotics. In experiments to determine whether agar augments the inhibitory effect of other antibiotics, concentrations of the antibiotics several times greater than their minimum inhibitory concentrations (as determined by the gradient plate method) were used. The results are summarized in table V. Both tetracycline and oxytetracycline were not appreciably affected due to the presence of agar. Streptomycin inhibition was reversed. Inhibition was appreciably augmented in the case of chloramphenicol and less so with penicillin.

DISCUSSION

The effect autoclaved agar has in augmenting the inhibition of *M. pyogenes var. aureus* by chlortetracycline is well established. The fact that washed agar [illegible]es

TABLE IV

Comparative Effect of Some Carbohydrates and Agar (3 Per Cent w/v) on Chlortetracycline Inhibition of M. pyogenes *var.* aureus *209P in Nutrient Broth*

Incubation period, hours	Survivors per ml. of medium containing:					
	Nil	Glucose	Sucrose	Dextrin	Starch	Agar
0	7.7×10^5	7.6×10^5	4.3×10^5	3.8×10^5	3.5×10^5	6.3×10^5
24	4.1×10^5	5.8×10^5	7.6×10^5	5.9×10^5	6.1×10^5	9.7×10^6

TABLE V

Comparative Effect of Agar (3.0 Per Cent w/v) on Inhibition of M. pyogenes var. aureus 209P by Different Antibiotics

	Survivors per ml.* after 24 hours at 37 C. in media containing:					
Agar	Chlortetracycline, 10 μg./ml.	Oxytetracycline, 10 μg./ml.	Tetracycline, 10 μg./ml.	Chloramphenicol, 600 μg./ml.	Penicillin, 130 units/ml.	Streptomycin, 10 μg./ml.
−	2.9×10^5	2.9×10^5	3.0×10^5	7.1×10^3	1.4×10^4	$<10^1$
+	1.1×10^6	2.2×10^5	3.0×10^5	$<10^1$	5.5×10^3	3.1×10^2

* Cells inoculated per ml. of each medium: 4.7×10^5.

not manifest this effect indicates that the factor(s) responsible for augmented inhibition is (are) water- and/or alcohol-soluble. Several possibilities for the observed effect exist. Growth substances associated with agar[10, 11] could increase the growth rate of the test organism and result in improved inhibition by the antibiotic; or, the agar may affect the availability of inorganic nutrients to the organism,[12] thus making it more susceptible to inhibition. The inactivation of the tetracyclines in vitro was reported to be noticeably prevented by serum, and this was ascribed to its polysaccharide content;[13] this could possibly account for the effect of agar.

The observation that agar did not augment inhibition by tetracycline and oxytetracycline suggests that structurally similar antibiotics may produce antibiosis differently. The increase in inhibition by chloramphenicol and the reversal in streptomycin inhibition brought about in the presence of agar is interesting.

SUMMARY

Autoclaved agar was found to augment markedly the inhibition of *Micrococcus pyogenes* var. *aureus* by chlortetracycline. The effect was more marked with unwashed agar than with washed agar, and at concentrations greater than 2 per cent. Sucrose, dextrin, and starch were found to reverse the inhibition. Inhibition of the test organism by chloramphenicol and, to a lesser extent, by penicillin was augmented by agar; the inhibition by tetracycline and oxytetracycline was not appreciably altered; the inhibition by streptomycin was reversed.

ACKNOWLEDGMENTS

Chlortetracycline (batch no. 45775-166) was a gift from Lederle Laboratories Division, Bombay, India. The award of an Imperial Chemical Industries (India) Fellowship by the National Institute of Sciences of India made part of this investigation possible.

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CARBOHYDRATE RESEARCH
17(1): 227-230, 1971

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Note

Chemical heterogeneity of the agar from *Gelidium amansii*

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(Received April 20th, 1970; accepted in revised form, July 17th, 1970)

Agars have been found to be mainly composed of two neutral sugar residues, D-galactopyranose and 3,6-anhydro-L-galactopyranose[1]. Smaller proportions of acidic components, such as sulfate[2], pyruvic acid[3], and uronic acid[4] residues have also been confirmed to be present in agars. Separation of agar into two fractions, one rich in neutral components and the other rich in acidic ones, has so far been achieved by acetylation followed by extraction with chloroform[5], precipitation with cetylpyridinium chloride[6], and precipitation with polyethylene glycol[7]. The former fraction, named agarose, has been shown to be a linear, nearly homogeneous molecule composed of alternating residues of the aforementioned two sugars[8]. However, the homogeneity and the structure of the latter fraction (agaropectin) has not been investigated in any detail except to show that pyruvic acid is bonded to D-galactopyranose residues[9]. It is very questionable whether the agar consists of only two polysaccharides having clearly different structures.

In order to obtain information on this point, the agar from *Gelidium amansii*, one of the most typical agarophytes found on the coastline of Japan, has been fractionated more completely by anion-exchange chromatography. First it was separated into four fractions (Fractions 1 to 4), each of which constitute approximately one fourth of the whole agar mixture. The compositions and yields of these four fractions are shown in Table I. The data indicate that the differences in proportions of the neutral sugars are very small, and the molar ratio of anhydrogalactose to galactose is approximately unity through each of the fractions. This observation suggests that a common structure having a rather homogeneous arrangement, possibly consisting of alternating residues of the two neutral sugars, is present in all agar fractions. By contrast, the proportions of the three acidic residues are remarkably different among the fractions, and it is noteworthy that the proportions of sulfate and uronic acid residues become gradually higher in the order of elution, whereas the proportion of pyruvic acid residue is highest in Fraction 3. Fraction 1 appears to be an essentially neutral polysaccharide, and Fraction 2 is intermediate in composition between Fractions 1 and 3 or 1 and 4. The yields of agarose and agaropectin from the same species of algae, as separated by extraction with chloroform, have been reported to be about 60 and 40%, respectively[8]. Comparing the yields and the compositions of the agar fractions separated by these two methods, agarose by the old method appears

to be a mixture of at least the Fractions 1 and 2 obtained in the present method, and agaropectin is a mixture of the Fractions 3 and 4.

TABLE I

COMPOSITION AND YIELD OF THE AGAR FRACTIONS FROM *Gelidium amansii*

Component	*Content* (%)				
	Agar	*Agar fractions*			
		1	2	3	4
Galactose[a]	47.1	51.0	45.6	43.0	42.8
3,6-Anhydrogalactose[a]	40.5	43.9	45.6	38.5	36.8
Uronic acid[b]	0.64	nil	0.09	0.30	1.39
Sulfate[c]	1.42	nil	0.77	0.94	2.36
Pyruvic acid[d]	2.28	0.16	0.71	5.98	1.05
Yield (%)		16	17	22	20

[a]Expressed as glycosyl residues. [b]Expressed as D-glucopyranosyluronic acid residues. [c]Expressed as SO_3. [d]Expressed as the free acid.

In order to clarify whether the fractions separated were homogeneous or not, Fractions 2 and 3 were subjected to gradient-elution chromatography. Fraction 2 showed a rather broad, but distinctly heterogeneous elution pattern, as revealed by the content of anhydrogalactose. Fraction 3 also showed a heterogeneous, and at the same time more sharply divided pattern, as shown in Fig. 1. The content of pyruvic

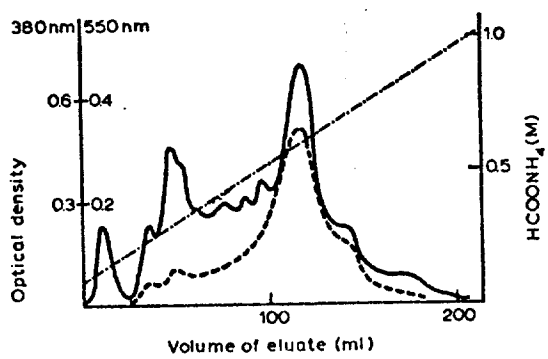

Fig. 1. Gradient-elution chromatography of the agar Fraction 3 from *Gelidium amansii* on a Dowex-1 X2 column. Content of pyruvic acid residue (------). Content of anhydrogalactose residue (———). Eluent concentration (-·-·-·-·-).

acid residues in the eluate was also determined, and is plotted against the elution volume in Fig. 1; this value also indicated heterogeneity in the agar, but the pattern was markedly different from the one obtained from the anhydrogalactose content. Fig. 1 thus indicates that elevation of the eluent concentration increases the content of pyruvic acid residues almost linearly, suggesting that Fraction 3 has marked chemical

heterogeneity. The maximum value of the molar ratio of pyruvic acid to anhydrogalactose residues is 0.45, indicating the presence in Fraction 3 of a polysaccharide containing on an average one pyruvic acid residue per tetrasaccharide unit.

All of the results given above accord with the conclusion that the agar from *Gelidium amansii* consists of a family of polydisperse polysaccharides that have a similar macromolecular structure, with continuously variable proportions of acidic substituents such as sulfate, pyruvic acid, and uronic acid residues. It is therefore necessary to reconsider the nomenclature of agar. The designation agarose may be retained for an essentially neutral polysaccharide consisting of repeated 4-*O*-β-D-galactopyranosyl-3,6-anhydro-L-galactopyranose residues, or agaran may be used instead. However, the designation agaropectin should be discontinued since its original meaning has now become ambiguous. Similar examples of a family of polysaccharides showing homogeneity by some criteria and heterogeneity by others are becoming increasingly common in the field of plant cell-walls and mucilages, for example, in pectic substances[10,11], the water-soluble arabinogalactan from *Codium fragile*[12], and carrageenan from some species[13]. Chemical heterogeneity of these polysaccharides in the cell-wall matrix will presumably include species- and tissue-specific characteristics. More-detailed investigations of these polysaccharides, particularly during cell differentiation and development, will provide an important clue to elucidation of their biological functions.

EXPERIMENTAL

Agar was extracted with hot water from *Gelidium amansii* harvested on the coast of the Izu Peninsula, and was purified by repeated precipitation with ethanol after a freezing and thawing procedure.

Fractionation of agar was performed by an ion-exchange chromatography on a column of Dowex-1 X2 (formate form, 200 to 400 mesh) at 80°. Agar solution (0.25%) in hot water (400 ml) was placed on the hot water-jacketed column (3.4 × 33 cm). The column was washed with water (600 ml), and the combined effluent and washings were concentrated and dialyzed against running water. The non-dialyzed polysaccharide (Fraction 1) was precipitated by addition of ethanol (4 vols.) in the presence of small proportion of sodium acetate, and was washed with acetone, and dried.

The polysaccharides retained by the column were eluted successively with 0.1M (700 ml) and M (900 ml) ammonium formate and 2.0M (1100 ml) ammonium salicylate solutions at pH 7.0 (Fractions 2, 3, and 4 respectively). The flow rates were 300 to 500 ml per h. The polysaccharides were isolated from the eluates by concentration, dialysis, and precipitation with ethanol, in the same manner as used for Fraction 1.

Rechromatography of Fractions 2 and 3 was performed on an analytical scale by using a smaller column (0.9 × 32 cm) for 0.1 to 0.25% sample solutions (3 ml). Concentrations of eluents (ammonium formate solutions) were raised through gradients from zero to 0.1M and 0.1 to M, respectively, during elution. The total

volume of eluents were 300 ml and every 3 ml of eluate was collected. The flow rate was 50 ml per h.

3,6-Anhydrogalactose was determined by the resorcinol method of Yaphe[14]. The content of galactose was calculated by subtracting the anhydrogalactose content from total hexose content as determined by the orcinol method of Hewitt[15]. Pyruvic acid was determined by the dinitrophenylhydrazine method of Katsuki *et al.*[16] after hydrolysis in 0.1M HCl for 4 h at 100°. Uronic acid was estimated as follows. Agar (10 to 15 mg) was partially hydrolyzed in 0.5M sulfuric acid for 2 h at 100°, neutralized with barium hydroxide, and centrifuged. The supernatant was passed through the columns (0.7 × 6.0 cm) of Dowex-50 X8 (H^+ form, 200 to 400 mesh) and Dowex-1 X8 (acetate form, 200 to 400 mesh) successively. Uronic acid-containing oligosaccharides retained in the latter column were eluted with M sodium acetate (pH 4.6, 20 ml) and uronic acid in the eluate was determined by the carbazole–borate method of Bitter and Muir[17]. Galactose and anhydrogalactose had been known to be slightly colored by the method, and the contribution of these sugars in the eluate to the optical density was corrected after estimating them separately by the orcinol and the resorcinol methods. Sulfate was estimated by the barium chloranilate method of Wainer and Koch[18] after hydrolysis in M hydrochloric acid for 5 h at 100°.

ACKNOWLEDGMENT

The author expresses his gratitude to Dr. S. Hirase, Kyoto Technical University, for helpful discussion.

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J. Assoc. Offic. Agr. Chemists
43: 624-632, 1960

Isolation and Detection of Gums in Frozen Desserts*

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The proposed promulgation of definitions and standards of identity for frozen desserts (1) dictates that increased attention be given to the detection and determination of gums in frozen desserts. This topic has been under consideration by this Association for 25 years. Cross (2) reported that there were no suitable methods at that time (1934) and undertook a study of gums in various products, including ice cream. The first report to the Association of a method that showed promise was made by Hart (3) who reported some success with a method for gums in cheese. He detected the presence of gums in authentic ice creams containing locust bean and tragacanth. This method was not studied collaboratively.

Official methods are available for the isolation and detection of gums in soft curd cheese, and in mayonnaise and French dressing (4, 5). With these methods the presence of gums has been detected by reactions of hydrolysis products after the gum has been isolated. Essentially, this isolation technique consisted in removal of fat with ether, precipitation of proteins, and final precipitation of the gum from aqueous solution with alcohol. Experiments conducted by previous Associate Referees showed the procedure would work on ice cream, but results of collaboration were poor. Failures to recover the gum material were frequent, and losses probably occurred when emulsions formed during fat extraction (6). Johnson (7) reported a method for isolating gums in cheese spread in which the problem of emulsions was overcome by using dioxane, which has the property of removing fat and water. This method had not been subjected to collaborative study. Using dioxane to remove butterfat from a laboratory ice cream worked exceptionally well when tried by the Associate Referee.

Chemical methods for identification of gums have been published (8, 9). Newburger, *et al.* (10, 11) published a technique for obtaining the infrared spectra of water-soluble gums. The latter method was chosen for study because of the specificity offered by the technique. A thin film of the gum is prepared and the infrared spectrum, *vs.* air, is determined.

Essentially, the method of isolation involves removal of fat with dioxane, precipitation of protein, and isolation and purification of the gum by alcohol precipitation. Obviously the gum must be rather pure

* Presented as the report of the Associate Referee at the Seventy-third Annual Meeting of the Association of Official Agricultural Chemists, Oct. 12-14, 1959, at Washington, D.C.

for identification and, for this reason, the method employs a triple precipitation as cleanup. Dilute HCl (1 + 1) is used instead of acetic acid (4, 5) because carbonyl-type absorption is characteristic of gums. "Salting out" is accomplished with saturated NaCl solution instead of $KAl(SO_4)_2$ solution because NaCl is transparent in the infrared while ionic sulfate shows absorption in the 9 μ region. In all probability these interferences would be present in small amounts; however, time did not permit a study of the effects that might be produced in the spectra.

METHOD

Reagents

(a) *Dioxane.*—Technical grade is satisfactory. (*Caution:* Vapors are obnoxious and harmful.)

(b) *Trichloroacetic acid solution.* — 50%. Freshly prepared as needed from non-hydrolyzed reagent.

(c) *Organo-silicone compound.*—To prepare non-wettable surface. Desicote ® (Scientific Instruments Division, Beckman Instruments, Inc., Fullerton, Calif.) has been found satisfactory.

Apparatus

(a) *Infrared spectrophotometer.*—Recording, for operation in 2–15 μ region.

(b) *Water-repellent plate.*—Wash 3–4" glass square thoroughly with detergent, rinse, and dry with towel. Dip glass rod in organo-silicone compound and streak adhering liquid across plate. Repeat streaking several times. Rub plate with lens paper to distribute evenly; then rub with clean lens paper to remove excess. Plate is now ready for use. Wash plate with cold H_2O and dry with towel after each use. Plate can be reused as long as it remains nonwettable.

Preparation of Sample

Weigh 50 g sample of frozen dessert into 250 ml centrifuge bottle and heat to 60° in H_2O bath. Add 150 ml dioxane, shake vigorously 2 minutes, and centrifuge 10 minutes at 1800 rpm. Decant and discard supernatant. Add 30 ml ether and shake vigorously to break mass at bottom, using rod if necessary. Decant ether and repeat ether wash once. Heat in H_2O bath to remove residual ether. Add 30 ml 80° H_2O and shake vigorously 2 minutes to dissolve or disperse residue.

Separation of Gum

Add 20 ml 50% trichloroacetic acid and heat to 60° in H_2O bath. Shake 1 minute and centrifuge 10 minutes at 1200 rpm. Decant solution through fast folded paper into second centrifuge bottle, and discard residue.

Fill centrifuge bottle with alcohol, add 1 ml saturated NaCl solution, mix, and let stand until coagulation occurs. If precipitate does not form, gums are absent. (Let opalescent solutions stand overnight to facilitate precipitation. Centrifuge and discard if no precipitate is present.) Centrifuge 10 minutes at 1800 rpm and immediately decant and discard supernatant.

Purify precipitated gum by adding 30 ml 80° H_2O and shake vigorously to dissolve or disperse precipitate. Fill centrifuge bottle with alcohol, add 1 ml saturated NaCl solution and 1 drop HCl (1+1), mix, let precipitate coagulate, centrifuge 10 minutes at 1800 rpm, decant, and discard supernatant. Repeat purification step twice.

Chemical Detection of Gum

(a) *Before infrared identification.*—Scrape small amount of precipitated gum from centrifuge bottle with spatula and transfer to 50 ml beaker with about 10 ml hot H_2O. Add 2 ml HCl and boil 5 minutes. Neutralize to multirange indicator paper, using 30% NaOH solution first and completing with 10% NaOH solution. Remove paper and continue as in 15.147, beginning ". . . add 5 ml Benedict's solution."

(b) *Testing large amount of precipitate.*—If infrared spectrum is not desired, spectrum indicates presence of 2 or more gums, or precipitated gum will not form film (as with karaya), proceed as in 15.147, beginning "Add to residue in tube . . ."

(c) *Infrared identification.*—(May not be applicable to mixtures of gums.) Dissolve or disperse residue in 35 ml H_2O. Prepare film as below, obtain infrared spectrum against air, and compare with spectra of reference gums.

Preparation of Gum Films

Place H_2O repellent glass plate over 2" opening on steam bath. Pour enough of aqueous gum solution on plate to form circle about 2" diameter. (Volume required to produce film of sufficient area to block sample beam and of suitable thickness to produce characteristic spectrum varies with nature of

gum and its concentration.) Heat plate until film is dry and remove film with forceps. If film sticks to plate, remove with razor blade or tissue lifter. Transfer film to beaker and dry at 100° for 15-30 minutes. (Excessive heating may char some gums.) Place piece of film between 2 salt plates and obtain infrared spectrum against air.

Preparation of Reference Gum Films

Disperse 0.2 g gum in 30 ml 80° H_2O and add 20 ml 50% trichloroacetic acid solution. Continue as in *Separation of Gum*, beginning "Shake 1 minute and centrifuge 10 minutes at 1200 rpm."

Collaborative Study

Each collaborator was sent seven bottles of dry mix, two tubes of butterfat, and seven reference gums (guar flour gum, locust bean gum, Irish moss, pectin, gum arabic, agar, and sodium carboxymethylcellulose). The dry mix contained 5.7 g nonfat dry milk solids, 6.0 g sucrose, and 1.5 g dextrose. Five of the bottles also contained 0.2 g of a gum equivalent to 0.4% gum in the collaborative ice cream mix. (The proposed standards permit 0.5% gum.) Two of the bottles were marked as practice samples: "With added gum" and "Without gum." The other five bottles were identified by number only. The collaborators were instructed as follows: "Transfer 6 g butterfat and one bottle of dry mix to a 250 ml centrifuge bottle. Wash the bottle with a total of 31 ml hot (80°) water, and transfer washings to the centrifuge bottle. Shake vigorously 2 min. Additional warming and shaking may be necessary to produce uniform emulsions. This will simulate 50 g ice cream. Proceed with analysis, beginning, "Add 150 ml dioxane . . ." [This mixture is within the limits described by Sommer (12)]. Collaborators were not asked to perform the chemical tests for presence of gums.

The procedure for obtaining films of the gum material isolated was editorially changed after the method was sent out for collaboration. Instructions were reworded to prevent ambiguity since one collaborator submitted results that showed he used the entire amount of gum isolated to prepare the films. These films showed complete absorption in some areas; however, the unknowns were correctly identified. Two collaborators had previously reported correct results with excellent spectra before the change was issued.

Results and Discussion

Spectra submitted by collaborators closely parallel those for locust bean, Irish moss, pectin, gum arabic, and sodium carboxymethylcellulose (see Figs. 1 and 2). Results of collaboration are shown in Table 1. Except for Collaborator 8 all collaborators isolated all gums and each identification was correct with the exception of collaborative Sample 3, which contained locust bean gum. Most collaborators stated that locust and

Table 1. Collaborative results for identification of gums in ice cream mix[a]

Sample	Gum Present	Collaborator 1	2	3	4	5	6	7	8	9
With added gum	Sodium carboxymethylcellulose	x	x	x	x	x	x	x	x	x
Without gum	—	x	x	x	x	x	x	x	x	x
No. 1	—	x	x	x	x	x	x	x	—[d]	x
No. 2	Arabic[b]	x	x	x	x	x	x	x	—[d]	x
No. 3	Locust bean[c]	Guar or locust	Guar or locust	Guar	x	x	Guar, possibly locust	x	Locust-guar	Guar
No. 4	Irish moss	x	x	x	x	x	x	x	x	x
No. 5	Pectin	x	x	x	x	x	x	x	x	x

[a] x = Correctly identified. [b] Sometimes called acacia gum. [c] Sometimes called locust seed gum, carob bean gum, or St. John's Bread. [d] See explanation under *Results and Discussion*.

guar gave practically identical spectra. This difficulty was to be expected. Newburger, *et al.* (11) report: "The spectra of locust bean and guar flour gums are indistinguishable. However, some authorities believe that these gums are identical or at most differ in the proportions of substances which they contain." Ewart and Chapman (8) report: "Locust bean and guar gums give identical reactions and cannot be distinguished on the basis of these (chemical precipitation) tests." The Associate Referee found that the two untreated gums had identical spectra, but treating the gums as in the proposed method produced marked differences in the 10 to 10.5 μ region. Guar gum (Fig. 1) showed a minor peak at 10.3 μ; locust bean (Fig. 1) produced a flat plateau between 10 and 10.5 μ. Collaboration disproved this as a means of differentiation. Some collaborators obtained films for locust bean that also showed a minor peak at 10.3 μ.

The samples marked "Without gum" and "No. 1" were identical. Collaborator 8 reported, "powdery precipitate formed which would not give a film" for Sample 1. It is presumed that this material was protein. Possibly not enough trichloroacetic acid was added or the temperature might have been too low to effect good coagulation of the protein. A gum was isolated by Collaborator 8 for Sample 2 and the spectra were submitted; however, no identification was made. He reports: ". . . films for the reference gums were satisfactory except in the case of acacia. The film could not be removed from the glass plate. The film chipped off in fine flakes and could not be used. This was also true of collaborative Sample 1. The film from collaborative Sample 2 was also difficult to handle."

Since this collaborator did not place his films between salt plates, but rather used a cardboard holder with windows, it is presumed this is the reason he did not submit a spectrum of reference acacia. The spectrum he submitted for collaborative Sample 2 could be superimposed on spectra submitted for Sample 2 by other collaborators who used the same model instrument as Collaborator 8.

One collaborator reported Sample 4 contained agar when it actually contained Irish moss. His reference spectra and the spectrum obtained from Sample 4 were given to an analyst in this laboratory who was unfamiliar with the collaborative study or the incorrect identification. This analyst correctly identified the spectrum of Sample 4 as Irish moss. For this reason the reference spectra and spectra obtained from Samples 3 and 4 were returned to the collaborator for re-evaluation. His original identification of Sample 3 as locust bean gum was correct. He subsequently identified Sample 3 as guar gum and Sample 4 as Irish moss. He reported choosing Irish moss on the basis of the spectral region between 10 and 12 μ. This was the same area used by the analyst in this laboratory to distinguish between agar and Irish moss.

The spectra obtained from films of untreated guar, locust bean, pectin, agar, and tragacanth gums (Figs. 1 and 2) do not differ materially from the spectra obtained from films of the gums recovered in the isolation and reference procedures. The only differences were in the relative heights of absorption peaks. Possibly this can be explained by the fact that gums are natural products and contain many compounds. Slight changes in technique between analysts may isolate more or less of a particular compound, giving rise to the differences in relative heights of absorption peaks. Gum arabic (Fig. 2) and psyllium seed gum (Fig. 2) produced essentially the same spectra for treated and untreated material except for slight changes in carbonyl-type absorption in the 6 μ region. Sodium carboxymethylcellulose (Fig. 2) varied considerably in the 5.5 to 8.5 μ region for the treated and untreated gums. The dotted areas indicate the trace for the untreated gums in Fig. 2 (arabic, sodium carboxymethylcellulose, and psyllium seed).

The collaborators were asked to report what type of device was used to hold the films in obtaining the spectra. Two used the conventional liquid cell holder (demountable type), and one a holder normally used in the mull techniques. Collaborator 8 placed his films between two pieces of stiff paper with a window cut to allow passage of the

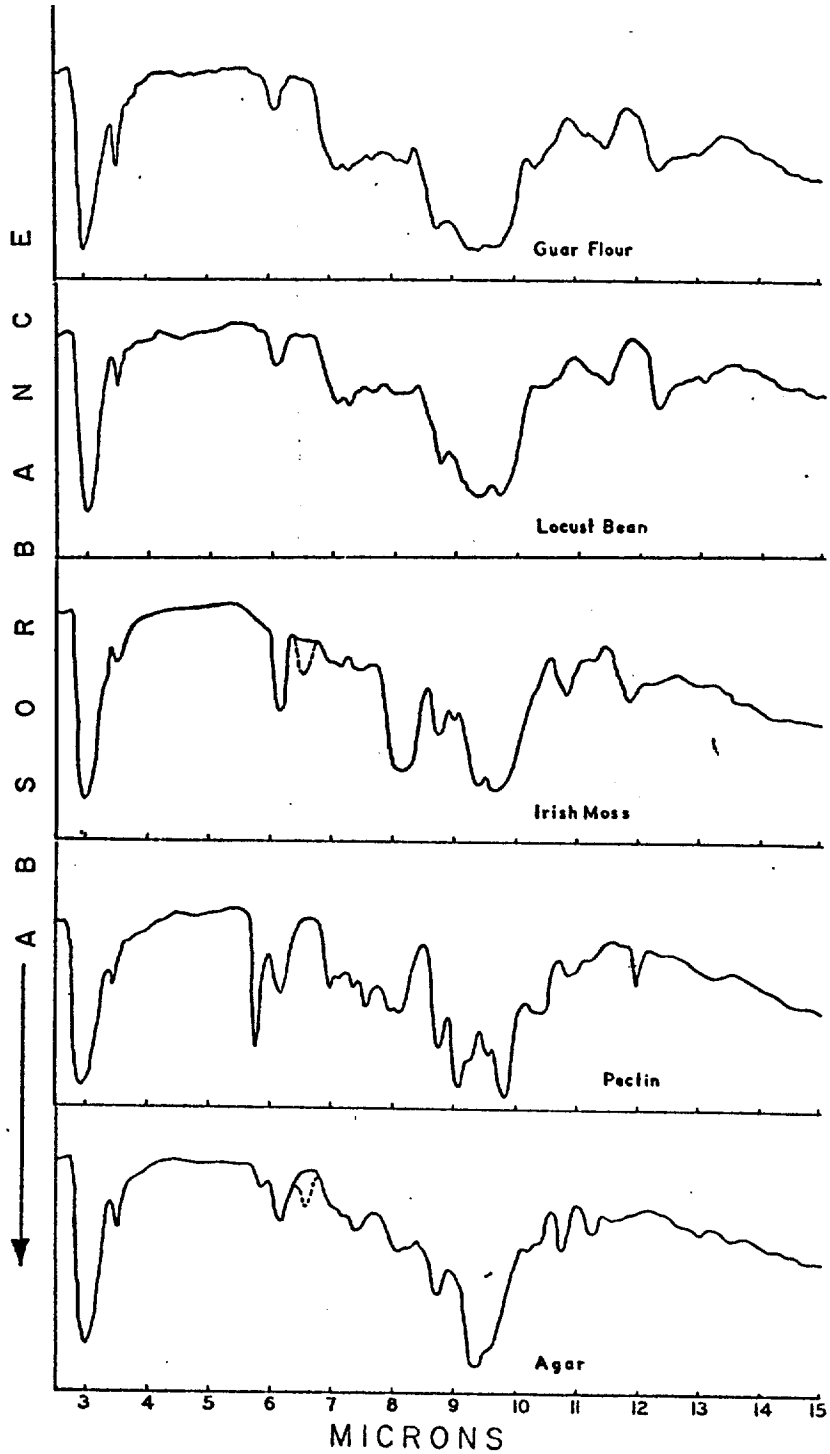

Fig. 1—Spectra of gums isolated by the method.

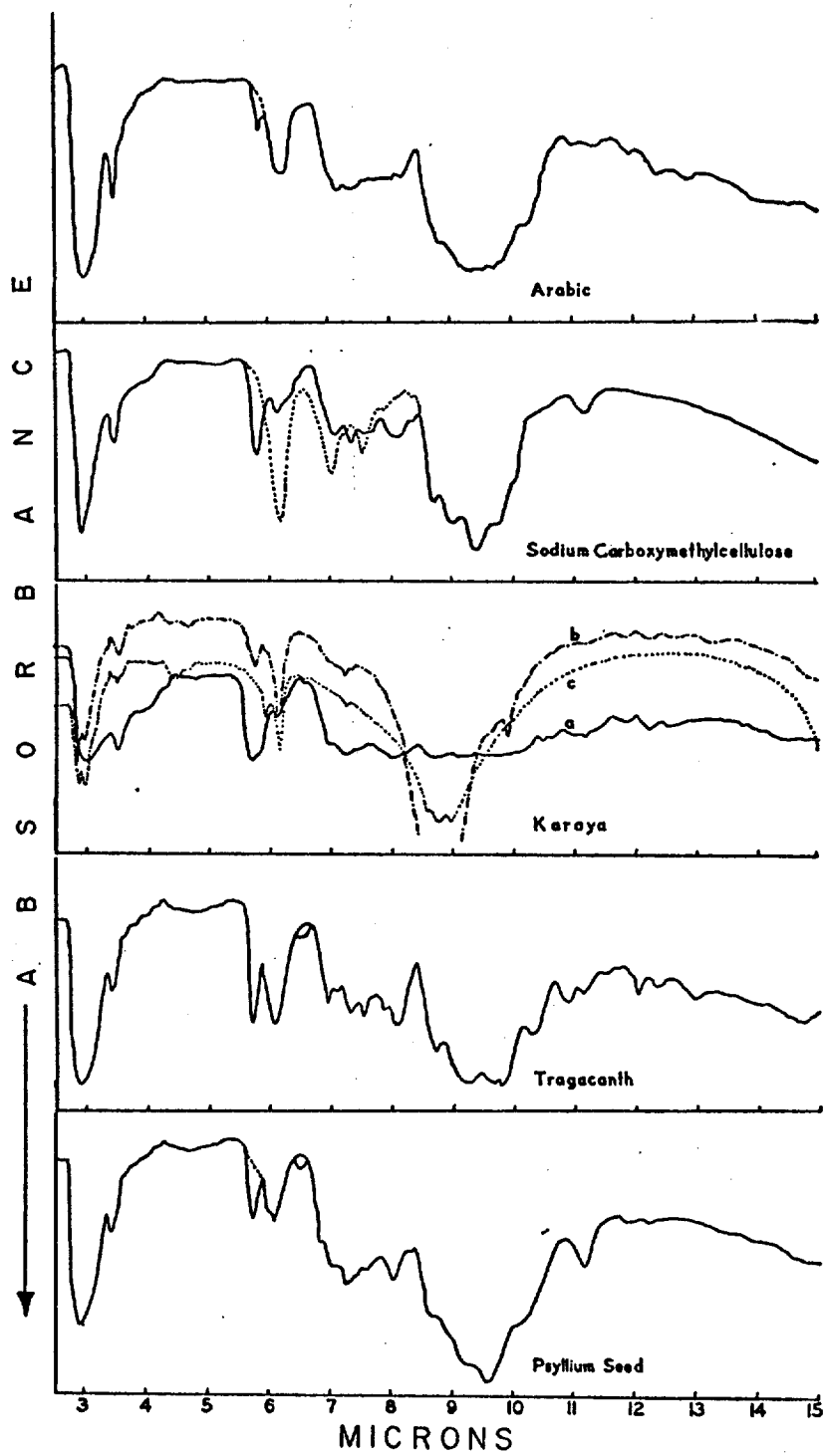

Fig. 2—Spectra of gums isolated by the method.

beam. While this technique is successful with well-formed films it cannot be used with the brittle, flake-type film such as gum arabic produces. The other collaborators used a device similar to the one described by the Associate Referee in his instructions. This device holds a piece of film between two salt plates with tension supplied by rubber bands. It is constructed of pressed board and eye screws. Samples can be changed quickly but it tends to damage portions of the film not covered by the salt plates. If no tension is put on the salt plates, films may fall out of the holder. Pressure may fracture brittle films, cause multiple reflections, and thereby give "jitters" to the recorder pen. While undesirable, this type of spectra does not negate identification since gums are natural products containing many compounds and, because of this, the absorption peaks are broad and an even pen line is not necessary for differentiating between spectra.

Experimental Work

A minor absorption peak at 6.5 μ occurred in each study of Sample 4, Irish moss, which did not appear in the spectrum of the reference gum. This absorption also occurs to a much smaller degree in agar, tragacanth, and psyllium seed gums isolated from the collaborative ice cream mix. (See dashed areas of Figs. 1 and 2). In order to study the nature of this peak the Associate Referee added Irish moss to butterfat, to sucrose, to dextrose, and to nonfat dry milk solids. The peak at 6.5 μ occurred only in the film of Irish moss isolated from the milk solids. A second protein precipitation did not remove this peak and produced an extremely poor, brittle film. The spectrum of isolated Irish moss that had been added to pure casein showed a minor absorption at 6.5 μ but not as intense a one as that from the milk solids. Conditions in this experiment were not exactly the same, however, since the casein was in large granules. It could not be reduced by grinding and, therefore, had to be dissolved in a 1% NH_4OH solution with the Irish moss. Irish moss was isolated from two other commercial brands of "instant" nonfat dry milk and these spectra also showed absorption at 6.5 μ. This type of study has not been conducted on agar, tragacanth, or psyllium seed gum.

A commercial "ice milk" frozen dessert was analyzed by the proposed method. The material isolated formed a film and the infrared spectrum was obtained (Fig. 3a). This spectrum was quite similar to that of tragacanth in the 8.5 to 11.5 μ region. From 11.5 to 12.5 μ the spectrum resembles guar or locust bean. The doublet effect between 5.5 and 6.5 μ occurs with about the same relative intensity as in psyllium seed and tragacanth. None of the other gums studied showed this doublet effect.

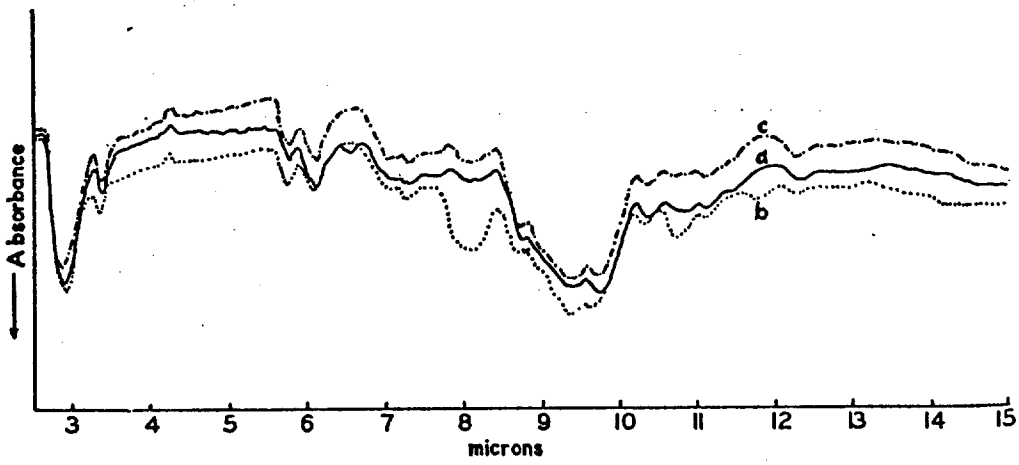

Fig. 3—Spectra of (a) gum material isolated from commercial "ice milk," (b) 1:1:1 locust bean, Irish moss, and sodium carboxymethylcellulose, (c) 1:1 locust bean and sodium carboxymethylcellulose.

The manufacturer of the ice milk said that the gum material used was supposed to contain a 1:1 mixture of locust bean and Irish moss to which he added sodium carboxymethylcellulose in an amount to make the final gum concentration a 1:1:1 mixture of sodium carboxymethylcellulose, locust bean, and Irish moss. A reference spectrum was prepared, using the 1:1:1 ratio (Fig. 3b). This spectrum showed strong absorption at 8 μ due to sulfate absorption in the Irish moss and slight absorption at 11.7 μ—also characteristic of Irish moss. A spectrum was prepared of sodium carboxymethylcellulose and locust bean in 1:1 ratio (Fig. 3c). This spectrum matched that of the gum material isolated from the ice milk. The strong absorptions at 8 μ and 11.7 μ were missing, indicating that no Irish moss was present in the ice milk. A sample of the commercial stabilizer used by this firm was obtained. The gum material was isolated and identified as either guar flour or locust bean gum. Information obtained about the formula for this stabilizing mix indicated it contained only locust bean gum and sucrose.

Chemical tests for the detection of gums were not studied collaboratively. The test with Benedict's solution (15.144(a)) for the detection of gums has been adopted as official (13). This test, in the modified form described in this report, was applied to Irish moss, tragacanth, psyllium seed agar, and the material isolated from the commercial "ice milk" dessert. Karaya isolated from the collaborative ice cream mix was tested by 15.147 (4). All tests were conducted by the Associate Referee and classified positive on the basis of the presence of a yellow, orange, or red precipitate.

The remaining gums permitted by the proposed standards, psyllium seed, guar, oat, tragacanth, karaya, agar, and the alginates were not subjected to collaborative study. The isolation and identification of these gums, with the exception of oat flour gum and the alginates, were studied by the Associate Referee. Each of the gums, except karaya, was isolated and each produced a film suitable for infrared identification (Figs. 1 and 2). Karaya, when isolated from the collaborative ice cream mix, produced a granular precipitate that formed a film with difficulty. The spectrum of this film was obtained (Fig. 2, Karaya b). This material was also ground and the spectrum was obtained by the KBr wafer technique (Fig. 2, Karaya c). These spectra were completely different from the spectra of the reference gum (Fig. 2, Karaya a). Karaya does not form a true solution, and most of the gum is probably lost in the protein precipitation step of the method. Hydrolysis may also be a factor. However, the presence of a gum in a frozen dessert containing karaya can be detected by 15.147 (4). Gelatin, also permitted in the proposed standards, is removed in the protein precipitation. A method for detecting alginates in chocolate products (14), including chocolate ice cream, has been adopted as official (15) by the Association. No oat flour gum was available to the Associate Referee for study.

Summary and Conclusions

A method has been described for the isolation, detection, and identification of gums in frozen desserts. The isolation and infrared identification of five singly occurring gums—locust bean, Irish moss, pectin, arabic, and sodium carboxymethylcellulose—was studied collaboratively with excellent results.

Agar, tragacanth, psyllium seed, and guar gum can also be detected and identified by the method, but they have not undergone collaborative study.

Guar flour gum and locust bean gum cannot be distinguished by this infrared technique. Karaya gum cannot be identified when isolated by the proposed procedure. The presence of a gum in a frozen dessert containing karaya (and/or other gums) can be detected by chemical tests. Brief study indicates that Irish moss, and to a lesser degree agar, psyllium, and tragacanth, binds some material from milk solids that cannot be precipitated by the protein precipitation method described. This material absorbs at 6.5 μ and is quite possibly nitrogenous in nature.

A commercial sample of frozen dessert was studied by the Associate Referee in which he detected the absence of one of the

gums reported to be a constituent of a mixture of three gums.

Recommendations

It is recommended[1]—

(1) That the method of isolation of gums in frozen desserts be adopted as first action.

(2) That the detection of agar, tragacanth, karaya, guar flour, psyllium seed, and oat flour gums in frozen desserts be studied collaboratively.

(3) That methods for separation of mixtures of gums be investigated.

(4) That quantitative methods for gums in frozen desserts be investigated.

Acknowledgments

The Associate Referee wishes to express gratitude to M. J. Gnagy, General Referee on Gums in Foods; and to J. T. Welch, former Associate Referee, for assistance in developing the method; to C. Comstock, Horton and Converse, Los Angeles, who supplied some of the gums used in the study; and to the following collaborators, all of the U.S. Food and Drug Administration: T. E. Byers, E. C. Pranskaitis, P. S. Jorgensen, B. W. Rehn, J. F. Weeks, C. E. Beisel, S. Nesheim, L. F. Schneiderer, A. I. Kleks, and M. J. Gnagy.

[1] These recommendations were approved by the Referee and Subcommittee C and were adopted by the Association. See *This Journal*, 43, 130 (1960).

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RELEASE OF HISTAMINE AND SERCTONIN FROM RABBIT THROMBOCYTES BY MEANS OF AGAR

Meuser & Vogt, Received 3 March 1968

Rabbits will release histamine and serotonin stored in them if they are incubated with plasma or serum which were previously "activated" by contact with agar (Humphrey & Jaques, 1955). The amine liberator apparently forming in the plasma is regarded as a substance similar to anaphy_latoxin (Giertz, Reutter & Schmutzler, 1964).

Together with Poppe & Schwoerer, we attempted to make use of the histamine release from rabbit thrombocytes as a biological test for the determination of anaphylatoxin. It was shown that agar will release histamine even in the absence of plasma. We will report on the more detailed investigation which showed that there are two effects from agar. Agar contains a direct and inherent and effective histamine releaser and agar furthermore also causes an indirect effect by activating the plasma and hence thus induces formation of a releaser.

METHODOLOGY

THROMBOCYTE SUSPENSION. All glass equipment coming into contact with blood or thrombocytes was siliconized. The rabbits were anesthesized by means of urethane (1 gm/kg, i.p.). By means of a polyethylene catheter tied into the a. carotis, the blood was

led into an ice-cooled measuring cylinder to which EDTA was simultaneously being introduced (0.02 ml of 10% neutralized Na_2 EDTA solution per ml of blood). Then the blood was centrifuged for 15 minutes at 2° (150 g). The supernatant platelet-rich plasma (30-50 ml) was pipetted off and again centrifuged for 15 minutes (300 g). While not all platelets were caused to precipitate on account of this second and relatively slow centrifugation, the deposited cells however were easily suspended again.

After decanting the plasma, the thrombocytes were rinsed with 40 ml of a 0.9 % NaCl solution (0.1% EDTA) and then with 40 ml of suspension solution (see below) and then floated in 8-25 ml of suspension solution. In order to separate thrombocytes clinging to each other, the suspensions were stirred for about 2 hours by means of a plastic rod, which rotated slowly, at 4°. The suspensions contained 200,000-600,000 platelets/mm3.

SUSPENSION SOLUTION (Humphrey & Jaques, 1955). The suspension solution used for the thrombocytes was of the following composition:

TYRODE solution	6 parts
Solution with 0.8% gelatin 0.8% sodium acetate & 25% NaCl	1 part
0.157 m sodium-phosphate buffer pH 7.6	1 part

PLATELET-FREE PLASMA. By centrifuging (2000 g, 30 min.) rabbit blood containing EDTA and by pipetting off the supernatant liquid, platelet-free plasma was obtained. 0.06 ml of 1% CaCl and o.01 ml of heparine were added to every ml of platelet-free plasma in order to bond the EDTA and nevertheless obtain non-coagulating plasma.

AGAR PREPARATIONS. A preparation containing all ingredients of agar , "Reinagar" (Behring), was used. Agarose (Mann) is an agar preparation containing only the neutral polysaccharides. A preparation of the acid agar ingredients ("Agaropektin") was obtained as follows: 1.5 gm of "Reinagar" were stirred in 150 ml of water at room temperature for 8 hours; the ingredients that had not dissolved were centrifuged off and the clear supernatant liquid was dialyzed with respect to water for 24 hours. Part of the retained material was dried and the residue was weighed in order to determine the Agaropektin concentration. The remaining solution was reacted with cooking salt to 0.9% and diluted with cooking salt solution to a concentration of 0.4 mg of Agaropektin/ml.

AMINE RELEASE BY MEANS OF AGAR. 2 ml of a platelet suspension were reacted with 0,05 ml of aminoguanidin of 4.10^{-5} g/ml in order to inhibit any histaminase that might be present and were incubated in a water bath for 15 minutes at 37° with 2 ml of the solution to be tested (incubated mixtures of agar with rabbit plasma or suspension solution, or these liquids alone). Then we cooled the preparations in ice water and separated them by means of centrifuging for 15 minutes at about 2° with respect to sediment and supernatant liquid. The platelet sediment was absorbed in 4 ml of suspension solution. 4 ml of 0.4 n perchloric acid were added both to the sediment and to the supernatant liquid, then centrifuging was resumed and the supernatant liquids were used for the determination of histamine and serotonin.

AMINE RELEASE BY FRACTIONATION. In principle the same process was used as for the study of the agar effect, only the mixing ratios were changed.

1 ml of Agaropektin solution was incubated with 2 ml of rabbit plasma or suspension solution for 30 minutes at 37°. Centrifuging followed and 2 ml of the clear supernatant liquid were hatched at 37° for 15 minutes with 1 ml of 0.9% NaCl solution, 1 ml of throbocyte suspension (about 600,000 cells/mm3) and aminoguanidin. Finishing as before.

AMINE DETERMINATION. The histamine was fluorometrically determined according to the method of SHORE, BURKHALTER & COHN (1959) in 4 ml each time of the centrifuged perchloric acid solutions. Each time 3 ml were reacted with 0.48 ml of 0.4 n NaOH and then were shaken out according to the method of UDENFRIEND, WEISSBACK & CLARK (1955) and processed further for the fluorometric determination of serotonin according to the same authors. Measurements were performed with a spectrophotometer "Fluoros--- FS 1".

RESULTS

AMINE RELEASE BY AGAR-BRED PLASMA AND PURE AGAR SOLUTION

The thrombocytes suspensions were divided into four sets of 2 ml each. The following were added: 1.2 ml of untreated rabbit plasma; 2.2 ml of plasma that had been incubated 30 minutes with 2.5 mg/ml of Reinagar at 37° and had then been centrifuged; 3.2 ml of suspension solution.

Table 1. Histamine and serotonin release from thrombocytes in various incubation media. Each preparation contained 2 ml of thrombocyte suspension with approximately 500,000 thrombocytes/mm3 and 2 ml of added incubation medium.

Running number	Incubation medium	histamine, ug/l released	histamine, ug/l in residual platelets	serotonin, ug/l released	serotonin, ug/l in residua platelets
1	plasma	0	4.8	0	3.6
2	agar-plasma	4.1	0.8	2.8	0.6
3	suspension solution	0.1	4.9	0.1	3.3
4	agar-suspension solution	4.2	0.8	2.9	0.6

4.2 ml of suspension solution were bred with Reinagar as in 2) and centrifuged. The preparations were incubated for 15 minutes at 37° and then centrifuged into supernatant liquid and platelet residue. Fig. 1 shows the absolute histamine and serotonin values in a typical experiment. In principle, the same result was obtained in 9 tests. Table 2 shows in summary form the average values of amine release as percent of the amounts present.

The values found corroborate that plasma bred with agar may release most of the amines contained in the thrombocytes. The release however will also occur when a pure suspension solution is pre-treated in the same manner with agar and then is made to influence the thrombocytes. Accordingly the amine-releasing agent does not seem to be formed in the plasma but rather be contained already formed in the agar itself.

Table 2. Histamine and serotonin release ($\bar{x} \pm s$) from thrombocytes in % of the total. Brackets indicate number of tests.

Running No.	INCUBATION MEDIUM	HISTAMINE REL.(%)	SEROTON. REL.
1	plasma	8 +- 10 (8)	4+-4 (8)
2	agar-plasma	79+-14 (8)	82+-14 (7)
3	suspension solution	9+-9 (8)	7 +- 11 (8)
4	agar suspension solution	84 +- 17 (7)	86 +- 4 (8)

EFFECTS OF PURIFIED AGAR FRACTIONS

Agar is a heterogeneous polysaccharide. In largest part it consists of a neutral, swelling-capable yet water-insoluble carbohydrate ("Agarose") and to a small part of water-soluble acid polysaccharide sulfates ("Agaropektin") (ARAKI,1987). Both ingredients may be extensively separated by stirring in cold water (HEIDELBERGER,1958).

Agarose or Agaropektin as the case happened to be were pre-incubated with suspension solution or with rabbit plasma for for 30 minutes (see Methods). Then the mixtures were centrifuged and the supernatant liquid was bred with thrombocyte suspension. Table 3 shows the amine release.

Table 3. Amine release by means of amine fractionating as % of the initial thrombocyte content (x +- s). Substances used:(agarose, agar, centrifuged off) as 0.4 mg of agaropektin, plasma as 2 ml of suspension solution, 150,000 cells/mm3 of thrombocytes (final concentration of preparation). Total volume: 4 ml. n=3.

Running number	Incubation medium	histamine release %	serotonin release %
1	suspension sol.	9 +- 2	10 +- 3
2	agar "	62 +- 3	57 +- 9
3	agarose "	10 +- 1	13 +- 3
4	agaropektin "	82 +- 11	79 +- 12
5	plasma	12 +- 0	12 +- 1
6	agar plasma	51 +- 1	61 +- 3
7	agarose plasma	17 +- 3	16 +- 3
8	agaropektin plasma	73 +- 8	75 +- 3

The values show that agaropektin is the directly amine-releasing substance in agar. Agarose will release no more amine than is the case for a suspension solution (spontaneous liberation). Agaropektin is more effective in salt solution than in plasma.

In order to determine which concentration of agaropektin suffices to release the amines, the test series shown in table 4 was carried out. It will be seen that already 8 ug/ml suffice.

Table 4. Amine releases from increasing concentrations of agaropektin.

Running number	agaropektin (final conc.)	histamine release %	serotonin release %
1	-	29	23
2	0.32 ug/ml	24	16
3	1.6 ug/ml	19	13
4	8.0 ug/ml	72	76
5	40.0 ug/ml	85	82

Table 3 further shows that following pretreatment with agarose, the plasma will be slightly histamine-releasing /which is lacking in pre-incubated plasma, and which does not belong to agarose; see and compare preparation 7 with preparations 5 and 3. The difference in values between preparations 7 and 3 is significant as regards histamine ($p < 0.05$). This histamine-releasing effect is reinforced if the plasma is pre-incubated with a ten-fold amount of agarose (table 5). Release of serotonin too is indicated.

The relatively large standard deviation of the values as regards histamine and serotonin released by agarose-treated plasma (table 5, # 7) may be explained by that plasma lacking any amine-releasing effect in one test. The difference between spontaneous liberation in normal plasmas and reinforced liberation in agarose-treated

plasmas nevertheless is significant ($p < 0.05$). In two test-series carried out later and not reported herein, the same phenomenon was observed. Possibly some rabbits lack a factor required for plasma agarose activation.

Table 5. Amine releases by agar fractionates of higher concentrations, (x +- s). Details as in table 3, but application of 4.0 mg of agar (agarose, agaropektin); n=4.

Running number	incubation medium	histamine rel. %	serotonin release %
1	suspension solution	10 +- 1	10 +- 2
2	agar suspen. solution	85 +- 1	89 +- 2
3	agarose "	12 +- 6	14 +- 1
4	agaropektin "	90 +- 3	91 +- 1
5	plasma	13 +- 1	12 +- 2
6	agar-plasma	86 +- 6	85 +- 6
7	agarose plasma	51 +- 26	44 +- 21
8	agaropektin plasma	83 +- 5	88 +- 4

DISCUSSION

The agar histamine-releasing effect mostly rests on the content of agaropektin (or on an agent dissolving together with the agaropektin fraction) which will directly attack the thrombocytes without plasma intervening. Probably this is a non-specific disorder as regards permeability (Lyse?), because the release of histamine and serotonin run in parallel. Damage is not to be viewed as a pure physical effect since salicylate will inhibit the release (unpublished). We are assuming that agaropektin will trigger a stationary thrombocytic, enzyme reaction which will alter the membrane.

Agarose may not directly release histamine. This is plausible

because it does not dissolve and therefore does not at all enter in contact with the thrombocytes. However, agarose does "activate" rabbit plasma and thus induces a thrombocyte-damaging agent. This mechanism is similar to anaphylatoxin formation. The latter too is initiated by agarose, but not by agaropektin (LYNCKNER & VOGT, 1966), and the product, AT, is a histamine releaser. No statement is presently possible as regards the formation of a histamine releasing agent in the rabbit plasma as does occur through AT formation in the rat plasma. The substance developing in the rabbit plasma lacks an intestinal contracting effect (HAINING, 1956; GIERTZ, REUTTER & SCHMUTZLER, 1964). This does not exclude however that it may occur in a similar reaction. AT-forming ferments were found also in the rabbit plasma (SCHWOERER, 1968).

Thanks are tendered to Professors Lendle and Friedberg access to the Pharmacological Institute of the Goettingen University where the fluorescence measurements were performed.

J. Immunol. Virus Res. Exp. Chemother.
50(4): 229-235, 1945

NUTRITIVE VALUE OF AGAR AND IRISH MOSS[1]

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(Received for publication, November 14, 1940)

The plants of the sea have always been considered an important reserve of food and raw material for industrial products which could be utilized in any emergency. Practical difficulties of harvesting, transporting, and handling these bulky plants have been hindrances in their utilization as raw material for industry. Food preferences and competition with land plants have mitigated against their use as foods except in the Orient and in Hawaii where many types of raw, pickled, and dried sea plants are items in the daily diet.

Members of the fishery industry of the United States were particularly interested in determining the nutritive value of some of the commonly available products derived from marine vegetation which are used as stabilizers in foods, and at their request this study was conducted. The experimental data for two of the products, agar and Irish moss, are reported in this paper.

Agar is the commercial name for the dried and partially bleached gelatinous extract of certain species of red algae. Tressler (1923) reported that the following varieties of algae are found in sufficient quantities on the California coast to be of commercial importance: *Gelidium corneum*, *Gelidium cartilagineum*, *Gracilaria confervoides*, *Euchemia spinosium*, and various species of *Tenax* and *Gigartineae*. Practically all of the world's supply of agar has come from China, Japan, Ceylon, and Malaysia. The California operators estimate that a steady annual crop of 1,000 to 2,000 tons of algae could be harvested. This amount would supply a considerable portion of the demand for agar.

The prepared agar is added to jellies and is used as a thickener of sauces, soups, and gravies. It is used as a stabilizer in some types of ice cream and is added to candies, pastries, and desserts. The grade of agar used in the feeding tests herein reported is commonly used as bacteriological media and contains a comparatively high percentage of vegetable gum.

Irish moss is also known as Chondrus chondrus and Carrageen and is the dried and bleached plant of *Chondrus crispus* (Linni) or

[1] This paper was presented at the meeting of the American Chemical Society at Detroit, Michigan, on September 12, 1940.

TABLE 1

Composition of Diets Used

Ingredients	Per cent by weight							
Agar	.....	5.0	10.0	20.0	30.0	.....	.....	.....
Irish moss	.....	.....	.....	.....	.....	5.0	10.0	20.0
Sucrose	27.0	25.0	22.7	18.4	14.5	25.3	23.8	20.6
Dextrin, tapioca	25.0	23.0	21.3	17.6	13.5	23.5	22.1	19.2
Lard	15.0 [1]	.....	.....	.....	.....	.....	.....	.....
Casein, technical	15.0 [1]	.....	.....	.....	.....	.....	.....	.....
Lactalbumin	5.0 [1]	.....	.....	.....	.....	.....	.....	.....
Brewer's yeast	5.0 [1]	.....	.....	.....	.....	.....	.....	.....
Wheat germ	2.0 [1]	.....	.....	.....	.....	.....	.....	.....
Cod liver oil	2.0 [1]	.....	.....	.....	.....	.....	.....	.....
Salt mixture, regular	4.0	3.7	3.4	2.8	2.2	1.5	1.0	.....
Salt mixture, No. 3	.....	.....	.....	.....	.....	1.7	1.4	.....
Salt mixture, No. 4	.....	.....	.....	.....	.....	.....	.....	0.7
Supplement value:								
Carbohydrate equivalent	.....	4.0	8.0	16.0	24.0	3.2	6.1	12.2
Mineral equivalent	.....	0.3	0.6	1.2	1.8	0.8	1.6	3.3
Excess moisture	.....	0.7	1.4	2.8	4.2	1.0	2.3	4.5

[1] Same amount used in all diets.

of *Gigartina mamillosa*. Plymouth county, Massachusetts, is the chief center of the production of Irish moss in the United States.

Irish moss is used in making blanc mange and other puddings, as a clarifying agent for beverages, and as a stabilizer of chocolate in chocolate-milk beverages. The Irish moss used in the feeding tests herein reported was especially refined for use in foods and contained less protein and ash than is common to most grades.

EXPERIMENTAL PROCEDURE

Male albino rats were allotted to experiment at an average initial live weight of 53 grams with a range of 48 to 58 grams. The animals

TABLE 2

Composition of Agar, Irish Moss, and Salt Mixtures Used

Ingredients	Per cent by weight				
	Agar	Irish moss	Salt mixture		
			Regular	No. 3	No. 4
Moisture	14.0	20.8	.....	.....	.....
Nitrogen-free extract	79.7	60.3	.....	.....	.....
Protein	2.5	1.6	.....	.....	.....
Ether extract	0.3	0.7	.....	.....	.....
Ash	3.5	16.6	.....	.....	.....
Calcium lactate	.....	.....	31.0	.....	.....
Dicalcium phosphate	.....	.....	11.0	30.0	8.0
Dipotassium phosphate	.....	.....	22.0	40.0	60.0
Potassium chloride	.....	.....	12.5	15.0	15.0
Sodium chloride	.....	.....	8.0	.....	.....
Calcium carbonate	.....	.....	7.0	.....	.....
Magnesium carbonate	.....	.....	3.0	8.0	8.0
Magnesium sulphate	.....	.....	3.7	1.5	.....
Ferric citrate	.....	.....	1.3	4.0	6.5
Copper sulfate	.....	.....	0.4	1.0	1.5
Manganese sulfate	.....	.....	0.1	0.5	1.0
Potassium iodide	.....	.....	0.01	0.01	0.02

were individually housed in screened cages fitted with screen floors. Food and water were fed *ad libitum* and the intake of each was recorded.

Groups of six rats each were allotted to be fed the control diet and diets containing approximately 5, 10, 20, and 30 per cent agar and 5, 10, and 20 per cent Irish moss (Tables 1 and 2). The 30-per cent level of Irish moss was not fed because of a lack of cages. These quantities were chosen arbitrarily but the higher levels probably represent from 10 to 100 times as much as is likely to be consumed in the daily diet. The food intake of the various groups was calcu-

lated to the equivalent dry-matter content of the control diet. The excess was added to the water-intake records.

Originally, a nine-week test was contemplated. In order to obtain accurate apparent digestibility coefficients representing the entire period, paper towels were spread under the screen floors and the feces were collected every two or three days. These feces samples from individual rats were kept in a cool place and later dried to determine the dry-matter content. Preliminary tests soon showed that the agar and Irish moss could not be readily extracted from the dried feces. Consequently, another sample was collected during the 10th week for the determination of agar, Irish moss, and dry-matter content. The data for gain in weight and food and water requirements cover the 10-week period, while the data on the digestibility of agar and Irish moss are limited to the 10th week.

The feces samples collected during the 10th week, less an aliquote for dry-matter determination, were individually extracted three times with petroleum ether to remove fats. They were then digested in boiling water for one hour while being agitated at high speed. Filter aid was added and the solution was filtered through a Büchner filter. The digestion procedure was repeated twice, after which the combined filtrates were precipitated with alcohol and permitted to stand overnight to insure complete precipitation. The precipitate which included the nitrogen-free extract of agar and Irish moss plus some protein and ash was dried, and aliquot samples per group were taken for protein and ash determinations. The apparent digestibility of agar and Irish moss was calculated from the intake and excretion of nitrogen-free extract. This procedure probably does not give an absolutely true answer but was considered to be the best approximation that could be obtained under the circumstances.

EXPERIMENTAL DATA AND DISCUSSION

In general, the rats grew very well and at the close of the 10-week period the mean live weight per group ranged from 245 to 389 grams. Only in the group fed 20 per cent Irish moss was there an excessive mortality. Three of the six rats in this group died during the first two weeks of the experimental period and it is quite likely that a change of bacterial flora in the intestinal tract to those types which more readily digest Irish moss is essential to survival. The necropsies were essentially negative. A much more laxative condition was noted in the rats fed the Irish moss than in those fed agar. The bulk was not so great but the feces were poorly formed, very soft, and smeary. The mean total excretion of dry matter per group during the 10-week period was 26 grams for

the control group; 38, 87, 106, and 191 grams for the groups fed agar; and 28, 61, and 36 grams for the groups fed Irish moss. The dry-matter content of the feces varied from 50 to 65 per cent.

The data show that the rats within any group grew uniformly (Table 3) since the coefficients of variation ranged from six to 15 per cent. Only two groups showed an approximately significant difference from the mean of the control group. The group fed 10 per cent agar gained in weight almost 20 per cent more than the control group, and those fed 20 per cent Irish moss gained about 30 per cent less. It is difficult to explain why the 10-per cent level of agar should permit the animals to grow faster unless there was a benefi-

TABLE 3

Daily Gain in Weight of Rats for 10-Week Period

Group designation	Number of survivors	Mean daily gain	Coefficient of variation	Mean difference from control	Per cent level of t
		gm.	*pct.*	*gm.*	
Control	6	3.99	13	.....	....
Agar					
5 per cent	6	3.53	6	0.46	[1]
10 per cent	6	4.80	8	0.81	5
20 per cent	5	3.95	13	0.04	[1]
30 per cent	6	3.70	10	0.29	[1]
Irish moss					
5 per cent	5	3.36	15	0.63	[1]
10 per cent	6	3.69	11	0.30	[1]
20 per cent	3	2.74	12	1.25	5

[1] Nonsignificant difference.

cial effect on the functioning of the intestinal tract. This difference was apparent even at the end of a year when the agar-fed rats had a mean weight of 737 grams as compared with 529 grams for the controls.

The data (Table 4) are interesting since they show that only the groups fed 10 per cent agar, and five and 10 per cent Irish moss were able to gain in weight without using significantly more food than the control group. Just why the five-per cent level of agar should cause a higher food requirement while the 10-per cent level did not cannot be explained at the present time. The mean apparent digestibility of the dry matter was depressed progressively with increasing levels of agar, and the 10-per cent level was no exception. This tendency was not apparent when Irish moss was fed.

The water requirement per gram gain in weight varies as may be expected (Table 5). It is interesting to note that although the

water requirement varied more within some groups than the food requirement, the rats fed the 20 and 30 per cent levels of agar had a very uniform water requirement which was significantly higher than that of the control group. The group fed 20 per cent Irish

TABLE 4

Food Required Per Gram Gain in Weight and Apparent Digestibility of Dry Matter for 10-Week Period

Group designation	Mean food required	Coefficient of variation	Mean difference from control	Per cent level of t	Mean apparent digestibility of dry matter
	gm.	*pct.*	*gm.*		*pct.*
Control	3.00	6	....	....	96.6
Agar					
5 per cent	3.31	3	.31	1	94.9
10 per cent	3.01	3	.01	[1]	90.5
20 per cent	3.53	6	.53	1	87.8
30 per cent	3.99	7	.99	<1	79.4
Irish moss					
5 per cent	3.23	7	.23	[1]	95.8
10 per cent	3.20	8	.20	[1]	91.8
20 per cent	3.53	8	.53	5	94.0

[1] Nonsignificant difference.

TABLE 5

Water Required Per Gram Gain in Weight for 10-Week Period

Group designation	Mean water required	Coefficient of variation	Mean difference from control	Per cent level of t
	gm.	*pct.*	*gm.*	
Control	4.91	18	.....	....
Agar				
5 per cent	5.51	19	0.60	[1]
10 per cent	4.80	11	0.11	[1]
20 per cent	6.50	3	1.59	2
30 per cent	8.23	5	3.32	<1
Irish moss				
5 per cent	5.98	16	1.07	[1]
10 per cent	6.80	16	1.89	2
20 per cent	9.46	19	4.55	6

[1] Nonsignificant difference.

moss needed almost twice as much water per gram gain as the control group. The actual total water consumption for the 10-week period roughly averaged 1,400 grams for the control group and those fed five per cent of agar and Irish moss; 1,600 grams for the group fed 10 per cent agar; 1,800 grams for those fed 20 per cent agar and

10 and 20 per cent Irish moss; and 2,100 grams for the group fed 30 per cent agar.

The 10th week served as a feces collection period in order to determine the digestibility of the agar and Irish moss. The data indicate the rather surprising conclusion that the level fed has no important effect on the apparent digestibility of the nitrogen-free extract of agar and Irish moss (Table 6). There was considerable variation within several groups but the food-intake data were taken from the same period as the feces were collected, and day-to-day fluctuations in food intake and feces excretion would have a greater effect during a short period of one week than for a longer period.

The coefficient of variation is extraordinarily large for the group fed 20 per cent agar with a range in apparent digestibility of five to 47 per cent and for the group fed 10 per cent agar with a range of 12 to 40 per cent. A reasonable variability was noted within the other groups. In general, the apparent digestibility coefficient of agar is about 28 per cent and that of Irish moss is 50 per cent.

Although increasing levels of agar and Irish moss in the diet had no definite effect on the apparent digestibility of the vegetable gums, the data (Tables 7 and 8) indicate the interesting conclusion that there was a significant decrease in the apparent digestibility of dry matter of the remainder of the diet. The variation within groups was very small and the depression of apparent digestibility coefficient almost reached 10 per cent in the extreme case. A significantly greater depression is shown (Table 8) within each series when increasing levels are fed, except for the 10-per cent agar group. In these studies the depression of apparent digestibility is not great but the trend confirms other researches which indicate that in times of scarcity it may not be entirely desirable in the interest of national economy to force dilution of foods with inedible or poorly digested roughage.

The studies reported herein are concerned only with the nutritive value of the vegetable gum and no attempt was made to determine their value as sources of vitamins and minerals when these and other types of products derived from agar and Irish moss are eaten as salads or added to jellies, soups, or other foods.

SUMMARY AND CONCLUSIONS

Male albino rats with an average initial live weight of 53 grams were fed individually for a 10-week period on a control diet supplemented with 5, 10, 20, and 30 per cent of prepared agar and 5, 10, and 20 per cent of Irish moss refined for food purposes.

TABLE 6

Apparent Digestibility of Agar and Irish Moss for Single-Week Period

Group designation	Mean apparent digestibility	Coefficient of variation	Mean difference within series	Per cent level of t
Agar	*pct.*	*pct.*	*pct.*	
5 per cent	35.7	28	.....	....
10 per cent	23.6	42	12.1	[1]
20 per cent	18.3	91	17.4	[1]
30 per cent	29.1	23	6.6	[1]
Irish moss				
5 per cent	51.8	29	.....	....
10 per cent	51.0	16	0.8	[1]
20 per cent	45.3	18	6.5	[1]

[1] Nonsignificant difference.

TABLE 7

Apparent Digestibility of Dry Matter of Diet Minus Agar or Irish Moss for Single-Week Period

Group designation	Mean apparent digestibility	Coefficient of variation	Mean difference from control	Per cent level of t
	pct.	*pct.*	*pct.*	
Control	95.22	0.9	.....	....
Agar				
5 per cent	92.10	1.2	3.12	<1
10 per cent	89.22	5.5	6.00	3
20 per cent	86.78	4.4	8.44	2
30 per cent	85.64	1.7	9.58	<1
Irish moss				
5 per cent	93.79	1.2	1.43	7
10 per cent	91.00	1.2	4.22	<1
20 per cent	87.42	0.5	7.80	<1

TABLE 8

Effect of Increasing Levels of Supplements on Apparent Digestibility of Dry Matter of Diet Minus Agar or Irish Moss for Single-Week Period

Group designation	Mean apparent digestibility	Coefficient of variation	Mean difference within series	Per cent level of t
Agar	*pct.*	*pct.*	*pct.*	
5 per cent	92.10	1.2	.....	....
10 per cent	89.22	5.5	2.88	[1]
20 per cent	86.78	4.4	5.32	6
30 per cent	85.64	1.7	6.46	<1
Irish moss				
5 per cent	93.79	1.2	.....	....
10 per cent	91.00	1.2	2.79	<1
20 per cent	87.42	0.5	6.37	<1

[1] Nonsignificant difference.

In general, the animals grew very well except those fed a diet containing 20 per cent Irish moss which suffered a 50-per cent mortality.

Only the groups fed the 10-per cent agar and 20-per cent Irish moss diets showed a statistically significant difference in mean daily gain in weight from the control group. The former gained about 20 per cent faster and the latter about 30 per cent slower.

The rats fed the 10-per cent agar diet, and the five- and 10-per cent Irish moss diets required about the same amount of food per gram gain in weight as the control group. Statistically significant is the fact that more food was required by the other groups.

The rats fed the 20- and 30-per cent agar diets, and the 10- and 20-per cent Irish moss diets required significantly more water per gram gain in weight than did the control group. Almost twice as much water was required by the rats fed the diet containing 20 per cent Irish moss.

The apparent digestibility coefficient of the nitrogen-free extract of agar is about 28 per cent and that of Irish moss is about 50 per cent irrespective of the level included in the diet.

There is a statistically significant progressive depression in the apparent digestibility of dry matter of the remainder of the diet as the level of agar or Irish moss is increased. This depression amounted to almost 10 per cent when the higher levels of agar and Irish moss were fed.

Only the nutritive value of the vegetable gum was studied and no attempt was made to determine the total food value of these or other types of products made from agar or Irish moss.

ACKNOWLEDGMENT

Sincere thanks are due Mr. J. Douglas Rollow, Jr., for assistance in the care of the animals and Dr. A. B. Steiner for assistance in the chemical phases of this study.

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THE RÔLE OF PATHOGENICITY-ENHANCING SUBSTANCES IN MICE INFECTED WITH SHIGELLA DYSENTERIAE (SHIGA) AND OTHER ENTEROBACTERIACEAE

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Received for publication June 28, 1944

As is the case with other microörganisms, the minimal number of *Shigella dysenteriae* required for a lethal intraabdominal injection of mice is considerably lowered if they are injected in a suspension of gastric mucin (Boivin and Mesrobeanu (1)). Olitzki and Koch (2) showed that mucin is also able to increase the pathogenicity of *S. dysenteriae* for white rats. Ørskov (3) reported that when *S. dysenteriae* is given in mucin, extracellular lysis in the peritoneal cavity of the mouse is absent and phagocytosis greatly inhibited; he suggests that mucin delays the mobilization of the normal apparatus of resistance. The purpose of the experiments reported here was to reinvestigate the mechanism of the mucin action in mice infected with *S. dysenteriae*, to compare the effectiveness of various mucin preparations as well as of fractions isolated from mucins of different origin, and to ascertain whether other substances exercise a similar effect on *S. dysenteriae* and related microörganisms.

EXPERIMENTAL

The strains "Bukarest R" and "Bukarest S" were kindly provided by Professor A. Boivin. Mucin in the form of scales (Burroughs Wellcome & Co., London) and Mucin comp. (Nordmark-Werke, Hamburg) were used; they are designated in the following as mucin W and N respectively. Five g of mucin were suspended in 100 ml of distilled water as described by Henderson (4). The suspensions were autoclaved and shaken before use. Mucin W gave a homogeneous solution, while mucin N contained much insoluble material. The bacterial suspensions were prepared from 20-hr broth cultures diluted with 5 per cent mucin suspensions, or with the corresponding quantities of plain broth.

The different action of mucin W and mucin N. Following the method of Boivin and Mesrobeanu (1), 0.05 ml of the required dilutions of broth cultures were suspended in 0.45 ml of 5 per cent mucin suspensions and injected intraabdominally into mice. The results are given in table 1.

Table 1 shows that the lethal dose, considering broth as 1, was reduced by one-half by mucin W and 200,000 times by mucin N; the enhancing power is thus dependent on the mucin used.

The effectiveness of fractions isolated from mucin W and N. The mucins were fractionated according to the methods described by Anderson and Oag (5). From 10 g of mucin the following quantities of the various fractions were isolated:

	M1a	M1b	M1c	M2	M3
	t		*t*	*t*	*t*
Mucin W	1.07	traces	0.05	0.88	0.01
Mucin N	0.23	traces	0.08	1.06	1.62

Mucin N contains more protein (fraction M2) and insoluble residue (M3), while mucin W contains more carbohydrate (fractions M1). Five per cent stock suspensions of each fraction were prepared. To 0.45 ml of each of these suspensions 0.05 ml of the required dilutions of broth culture were added and injected into mice. The minimal lethal doses were as follows: Cultures diluted with broth, 0.1 ml; with M1a derived from mucin W or N, 0.005 ml; with M2 from mucin W or N, 0.0005–0.00005 ml; with M3 from mucin N, 0.000005. M3 from mucin W was ineffective.

TABLE 1

The pathogenicity-enhancing effect of mucin W and N

Five mice in each test group

BROTH CULTURE DILUTED IN	QUANTITY OF BROTH CULTURE INJECTED	NUMBER OF DEATHS AFTER INJECTION OF	
		R-bacteria	S-bacteria
	ml		
Mucin W	0.05	1	4
	0.005	0	0
	0.0005	0	0
Mucin N	0.00005	5	5
	0.000005	2	4
	0.0000005	0	0
Broth	0.10	1	3
	0.05	0	0

This experiment shows that the protein-fractions M2 have greater enhancing power than the carbohydrate fractions M1a, and that the residual M3 from mucin N is the most effective fraction. It contained 40.5 per cent of inorganic substances, nearly all of it consisting of aluminum.

Activation of inactive substances by different adsorbents. The following experiments show that 0.3 per cent agar, which itself has only a weak effect, is activated by the addition of a 1 per cent suspension of kaolin powder (E. Merck). With bacteria suspended in 0.5 ml of this mixture the following minimal lethal doses were obtained: *S. dysenteriae*, 1.7×10^5 bacteria; *Escherichia coli*, 6×10^5 bacteria; *Eberthella typhosa* (strain O 901 and Ty_1), 1.2×10^5 bacteria; strain Ty_2, $1 \times 10_2$ bacteria. The minimal lethal dose of strain Ty_2 suspended in broth was 1×10^7; that of the other strains 1×10^8 bacteria.

Various absorbents were examined. The quantity of bacteria injected corresponded to 10 lethal doses in kaolin-agar. The results are summarized in table 2.

The table shows that agar alone enhances the pathogenicity of the strain Ty_2, but has no significant effect on the other strains. The various adsorbents were inactive when tested alone but could enhance the pathogenicity of all strains suspended in 0.3 per cent agar. Charcoal (Pharmacie central de Belgique) and $BaSO_4$ were inactive and failed to activate agar.

Glycogen (20 per cent) and starch (10 per cent) were inactive and could not be activated by kaolin.

Levan was isolated on sucrose broth from a strain of *Bacillus subtilis* according to a method employed by Aschner *et al.* (6). A 4 per cent suspension of this carbohydrate was in itself weakly active, killing 2 to 3 mice in each group, if the same quantities of bacteria were given as in the former experiment. Suspended together with 1 per cent kaolin, it produced a 100 per cent mortality with all bacteria used above. A similar summative effect was observed after addition of kaolin to a 5 per cent suspension of mucin fraction M1a.

TABLE 2

The pathogenicity of bacteria suspended in different substrates

Five mice in each test group. Total volume injected 0.5 ml

BACTERIA SUSPENDED IN	NUMBER OF DEATHS AFTER INJECTION OF STRAIN					
	Shiga R	Shiga S	$Typhoid_1$	$Typhoid_2$	Typhoid 0 901	*E. coli*
Broth	0	0	0	0	0	0
Talcum 1%	0	0	1	0	0	2
Fullers earth 1%	1	1	0	1	2	1
Norit A 2%	0	0	0	1	0	0
Kaolin 1%	1	1	0	0	2	0
Agar 0.3%	0	1	0	4	1	1
Agar 0.3% + talcum 1%	3	3	5	5	5	5
Agar 0.3% + Fullers earth 1%	5	5	5	5	5	5
Agar 0.3% + Norit A 2%	4	4	4	5	5	4
Agar 0.3% + kaolin 1%	5	5	5	4	5	4

THE MODE OF ACTION OF THE PATHOGENICITY-ENHANCING SUBSTANCES

The delay of resorption. It seems likely that the activation of agar by kaolin and other adsorbents is mainly effected by delaying absorption. Kaolin itself disappeared from the peritoneal fluid within a few hours after the injection and only few big lumps were found macroscopically, whereas masses of kaolin-agar were seen in the abdominal cavity even 5 hours after the injection. If the enhancing substances were given separately and the injections of the bacteria (0.05 ml broth culture of the Shiga-strain) followed after various intervals, the results shown in table 3 were observed.

The table shows that the period of time during which these substances are effective parallels the delay of the resorption from the abdominal cavity. The highly active mucin N remains in such a concentration after 6 hours that the infection still can be stimulated. Mucin W in the same quantity disappears

in less than one hour. However, if injected together with kaolin it retains its activity for 1 to 2 hours. Agar itself was ineffective after 1 hour, but given with kaolin it retained its activity up to 5 hours.

Adsorption of cells. If the peritoneal fluid was spread on a slide and stained with Giemsa, we observed a marked adsorption of white cells by kaolin as well as by agar and still more striking by kaolin-agar. The number of free cells counted in each field (magnification 800 fold) was as follows:

30 min after injection of broth 0.5 ml: 4.0
30 min after injection of 0.3 per cent agar 0.5 ml: 2.2
30 min after injection of 1 per cent kaolin 0.5 ml: 1.0
30 min after injection of 1 per cent kaolin + 0.3 per cent agar 0.5 ml: 0

A similar effect was observed if simultaneously large quantities of bacteria (Shiga R 1 $\times$ 10^9) were injected. The average number of white cells counted per field in the corresponding groups was: 7.2, 4.2, 2.2 and 0.4.

TABLE 3

The duration of effectiveness of various substances

Five mice in each test group

SUBSTANCE INJECTED	NUMBER OF DEATHS IN EACH GROUP, IF THE INJECTION OF THE BACTERIA FOLLOWED AFTER					
	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr
Mucin N 5% 0.5 ml	5	4	4	4	3	2
Agar 0.3% 0.5 ml	1	0	0	0	0	0
Agar 0.3% + kaolin 1% 0.5 ml	5	5	3	3	3	1
Mucin W 5% 0.5 ml	0	0	0	0	0	0
Mucin W 5% + kaolin 1% 0.5 ml	3	2	0	0	0	0
Mucin W 5% + kaolin 1% 0.2 ml	3	1	1	0	0	0
Kaolin 1% 0.5 ml	0	0	0	0	0	0

Prevention of bacteriolysis and phagocytosis. Dysentery bacteria injected intraabdominally into mice disappeared quickly from the peritoneal fluid. If 0.05 ml of broth culture were injected and after 1 to 3 hours groups of 5 mice received 0.5 ml of 5 per cent mucin N suspension, all the animals died. If the mucin was injected at a greater interval, no deaths occurred. Similar results were obtained with kaolin-agar; if it was injected later than 1 to 2 hours after the bacteria, no deaths occurred.

The experiments show that within a few hours the number of bacteria in the peritoneal cavity decrease so much that the subsequent injections of the virulence-enhancing substances were without effect. Direct microscopic observations were made to determine whether this was caused by bacteriolysis or by cellular action: Groups of mice received bacteria intraabdominally and after varying intervals the mice were killed, the peritoneal fluid spread on a glass slide, stained with Giemsa stain and examined. The summary of a typical experiment is given in table 4.

It will be noted that after an injection of 2.5 $\times$ 10^8 bacteria in broth the number of bacteria decreased and after 5 hours no microörganisms were found.

The peritoneal fluid at first contained chiefly lymphocytes, but after 3.5 hours numerous polynuclears appeared; at that time no phagocytosis was observed. It would seem, therefore, that the destruction of the bacteria was extracellular. In presence of agar the number of bacteria increased during 3.5 hours and then decreased. It appears that only during the first hours agar is present in sufficient concentrations to prevent bacteriolysis. In the presence of kaolin the number of bacteria decreased, but the destruction of the bacteria was somewhat slower than without kaolin. In presence of kaolin-agar a rapid increase began promptly after the injection followed by a progressive decrease. After 5 hours numerous polynuclears appeared but they were without apparent phagocytic activity.

TABLE 4

Number of bacteria and cells found in the peritoneal fluid after the injection of dysentery bacteria suspended in different substrates

Total volume 0.5 ml

NUMBER OF BACTERIA INJECTED	TIME AFTER INJECTION	NUMBERS FOUND IF THE BACTERIA WERE SUSPENDED IN							
		Broth		Agar		Kaolin		Kaolin-agar	
		B	C	B	C	B	C	B	C
	hr								
2.5×10^8	0.5	24.3	3.0	26.6	2.2	32.6	3.2	11.2	0.6
	1.0	13.8	21.8	22.8	6.2	13.4	5.6	77.0	3.6
	2.0	2.0	17.4	58.2	7.8	15.6	11.4	146.0	4.4
	3.5	0.5	68.0	72.8	6.4	3.0	39.6	50.6	5.6
	5.0	0	26.6	1.0	12.6	1.4	45.2	27.8	47.8
1.0×10^9	0.5	16.2	8.6	42.4	5.4	122.0	2.4	117.0	1.8
	1.0	34.8	7.2	140.4	4.2	138.8	2.2	127.4	0.4
	2.0	85.0	12.8	∞	2.4	68.2	3.6	200.0	7.2
	3.5	268.0	14.8	∞	29.4	17.4	2.6	∞	2.0
	5.0	9.0	0.1	∞	23.4	0	46.0	∞	3.2

Remarks: B = number of bacteria; C = number of cells per field; ∞ = number too large to be counted.

After the injection of 1.0×10^9 bacteria suspended in broth there was first an increase in number and then a drop. In the case of the kaolin, large numbers of bacteria were found during the first 2 hours after the injection, followed by a decrease to zero after 5 hours. However, in contrast to the experiment with 2.5×10^8 bacteria, the diminution seemed to have been effected mainly by cells. The bacteria suspended in broth were phagocytized 2 to 3 hours after the injection; 95 per cent of the polynuclears participated and on the average 6.8 bacteria phagocytized were counted per cell. In presence of kaolin, 92 per cent of the polynuclears contained bacteria and 8.1 bacteria were counted per cell. In presence of agar or kaolin-agar the number of bacteria increased from the beginning of the experiment on and no phagocytosis was observed.

It seems that the manner of the destruction of the bacteria in the abdominal cavity is determined by the number of bacteria injected. Smaller quantities

are removed chiefly by extracellular lysis, while greater quantities are removed—after initial multiplication—chiefly by cellular action. In the kaolin-agar suspension the agar represents the active principle; if the quantity of bacteria is large enough, agar without kaolin is able to insure their further multiplication. Kaolin alone had only a retarding effect on the antibacterial action and could not prevent the reduction of the number of bacteria whether by lysis or by phagocytosis.

Action of kaolin and agar injected separately. If the bacteria were injected together with agar or kaolin respectively and a second substance given separately either before or after infection, the results were similar to those obtained by simultaneous injection of the two substances. The kaolin or agar remained in the abdominal cavity after several hours in such concentrations that the injection of the other substance together with bacteria gave the same results as if they had been injected simultaneously.

SUMMARY

1. Two mucin preparations of different origin showed different degrees of effectiveness in enhancing the pathogenicity of intraabdominally injected *S. dysenteriae.*

2. Fractionation of these mucins showed that the active mucin (N) contained more of an insoluble fraction rich in inorganic substance, mostly aluminum, than did the less effective preparation W.

3. Kaolin, as well as other inert adsorbing powders, activates some carbohydrates. Agar showed a weak pathogenicity-enhancing effect, but was activated by kaolin and other adsorbing powders. Levan itself, as well as carbohydrate fractions (M1) from mucin, showed marked effects, which were enhanced by kaolin. Glycogen and starch were inactive and were not activated by kaolin.

4. The activity of the pathogenicity-enhancing substances was inversely proportional to the rapidity of their elimination from the abdominal cavity. Kaolin and other adsorbing substances retard elimination.

5. Injection of bacteria with kaolin or with agar preceded or followed by an injection of an adsorbent had the same effect as a combined injection of the two substances.

6. The following properties are important for the effect of pathogenicity-enhancing substances: long persistence in the abdominal cavity, ability to adsorb and destroy white cells and, as a consequence, to prevent phagocytosis. The effect is, therefore, not an enhancement of pathogenicity but a depression of the mechanism of natural resistance.

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ON THE IDENTIFICATION BY MEANS OF ELECTROPHORESIS AND CELLULOSE ACETATE FOIL DYEING OF GELS & THICKENING SUBSTANCES THAT ARE LEGAL IN SWITZERLAND.

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1. INTRODUCTION

Besides their gelling and thickening properties, gels and thickening substances used in food manufacture or processing also evidence another important characteristic, namely their ability to emulsify and disperse, thereby providing the explanation for their increasingly wide use as stabilizers in the food processing industry.

Article 443 bis of the Federal Food Regulations defines gels and thickening substances as being those capable of yielding aqueous jellies or aqueous, highly viscous solutions already when in low concentrations. (Concerning admissibility of use, see same document, 56, 110, -1956). Even though they are often being used as stabilizers, they are not designated as such in the food regulation. Within the scope of judicial planning concerning food processing in the EEC, namely admissible emulsifiers, stabilizers, gels and thickening substances (WEISS, 1966), stabilizers were defined as such substances suited for maintaining uniform dispersion of two or more immiscible substances. Most gels and thickening substances are included.

Food monitoring efforts have not been wanting to the end of developing practical testing methods for the analysis of gels and thickening substances. Identification is rendered difficult by the fact that these are high-molecular substances for which no easily executed and specific reactions are known, -- except for starch and the alginates. Furthermore they are used in food only in very minute quantities most of the time, because the desired thickening or stabilizing effect often is already achieved for concentrations under 0.5%. The viscometric method (LETZIG, 1934), based on an appreciable viscosity increase of aqueous solutions due to thickening substances, allows an overall indication of any presence of a thickening substance, but does not permit its identification. Various methods are known for qualitative evidence of gels and thickening substances, which among other methods are based on microscopic tests (CZAJA, 1962; BEYTHIEN & DIEMAIR, 1963), on flocculation reactions (LETZIG, 1959) or on paper chromatographic tests of their hydrolysis products (BECKER, 1956; SULSER, 1957; STOLL & PRATT, 1962), that is, of the corresponding monosaccharides. If the gels and thickening substances to be tested are in pure form, they may be easily tested most of the time under the microscope or by the flocculation reaction. If however their presence in food must be shown, in which there is an interfering substance such as egg white material, then the paper chromatographic test of the hyrolysis products is more promising than a flocculation

reaction. However there are cases in which a few gels or thickening substances yield similar sugar components after hydrolysis or where such sugars as glucose and galactose (for instance from lactose in milk products) are detected in the paper chromatographic test, so that the chromatogram will not yield an unambiguous answer. Methods for testing the identity of the purity have been disclosed in the literature (ORGANISATION MONDIALE DE LA SANTE, 1964, 1966). Concerning their numerous applications outside food processing, see also GLICKSMAN (1964).

The process of paper electrophoresis has proved itself for gelatin detection in dairy products (see this publication, 56, 110, 1965). However the cellulose acetate foils are preferable to paper as carrier material, since good separation may be achieved in little time and because the foils may be rapidly rinsed in view of little dye absorption (see section 2.4.1). We further made use of electrophoresis for the analysis of gels and thickening substances belonging to the polysaccharide group.

The described testing method further allows -- besides identification of gels and thickening substances -- quantitative evaluation by means of dye comparisons with solutions of known concentrations of the pertinent gels and thickening substances, the comparison dealing with color intensity. Our research has shown this simple and easily executed electrophoresis process followed by dyeing promises to be very useful in the analysis of gels and thickening means.

As regards food products, polysaccharides may be determined after removal of fatty and egg-white components by means of alcohol precipitation, which is followed by polysaccharide concentration and the subsequently described method.

2. ELECTROPHORESIS OF GELS AND THICKENING SUBSTANCES

2.1 The cellulose acetate foil as carrier substance

Filter paper is unsuitable for electrophoresis research in gels and thickening substances. When the gels and thickening substances being tested are made visible after electrophoresis-separation (see section 3.3), the filter paper reacts as a polysaccharide when the particular dyeing method is used, and it reacts positively. We tested glass fiber strips and cellulose acetate foils in lieu of filter paper. The latter proved particularly suitable. When testing gelatine too, the paper electrophoresis method proved very useful, and cellulose acetate foils were preferable to filter paper as carrier material, because the lesser dye adsorption allowed rapid rinsing of the foils (see figures 1a and 1b).

An important advantage of cellulose acetate foil electrophoresis consists in the shorter test duration with respect to paper electrophoresis, or about 15 to 30 minutes for microelectrophoresis (section 2.4.2), so that interferences or secondary effects during separation, such as evaporation of buffer liquid and the resulting undesired concentration increase are only of insignificant magnitude.

2.2 BUFFER SOLUTIONS

The conventional buffer solutions for paper electrophoresis may also be used as conducting electrolytic solutions for cellulose acetate foil electrophoresis. In general, less concentrated solutions are preferred for the latter process. We selected a borate puffer with a pH of 10 for the electrophoresis of the gels and thickening substances of the polysaccharide group, however the sodium-carbonate sodium-hydrogen-carbonate buffer is more suitable when testing gelatine (see section 2.4.3). The buffer solution concentrations are so adjusted that for a terminal potential of 200 volts, a current of less than 1 ma/cm of foil width is generated. Using both buffers demonstrated anodic migration in all gels and thickening substances that were tested, so that they must be coated on the cathode side at the beginning of the test.

2.3 RENDERING GELS AND THICKENING SUBSTANCES VISIBLE.

Following electrophoresis migration, the cellulose acetate foil is removed from the electrophoresis chamber and the position of the gels and thickening substances will be rendered visible by dyeing them. The various gels and thickening substances evidencing different chemical properties, four different dyeing methods are required (see section 3), which may be differentiated through dyeing because the gels and thickening substances migrate at the same rate under electrophoresis.

2.4 RESULTS

2.4.1 COMPARISON BETWEEN CELLULOSE ACETATE FOILS AND PAPER STRIPS

Pherograms I and II in fig. 1a show the results of our electrophoresis test with cellulose acetate foils (4 x 30 cm) in the Elphor-H electrophoresis chamber of GRASSMANN & HANNIG. Except for buffer concentration and the kind of depositing of the substance tested, test conditions are the same as for paper electrophoresis. The amounts of carbonate buffer with pH of 10 and ion size of 0.15 microns are diluted with the same volumes of distilled water. Deposition of substance tested takes place - not with a micropipette - but with a stamp consisting of two parallel platinum laminae absorbing each time 6 microliter of liquid.

It will be noted when comparing figures 1a and 1b that the cellulose acetate foil electrophoresis allows good separation between gelatine and milk eggwhite in much shorter a time than is possible for paper electrophoresis (3 hours in lieu of 14). Furthermore, the foils may be rinsed when dyeing much faster also (20 minutes in lieu of 2 hours.

2.4.2 MICRO-ELECTROPHORESIS OF GELATINE WITH CELLULOSE ACETATE FOILS

The BECKMANN-SPINCO microzone electrophoresis system allows executing simultaneously 8 separations for probe quantities of 0.25 microliter solution of substance , corresponding to 2-10 microgram of the substance tested, on one cellulose acetate foil of 5.5 x 14 cm (see section 4). Deposition of substance tested is performed by means of a microstamp. This allows reducing separation time to 15-30 minutes. Normally the separation time

on a cellulose acetate foil 4 x 30 cm in size and with the Elphor-II electrophoresis chamber of GRASSMANN & HANNIG amounts to about 3 hours (see section 2.4.1)

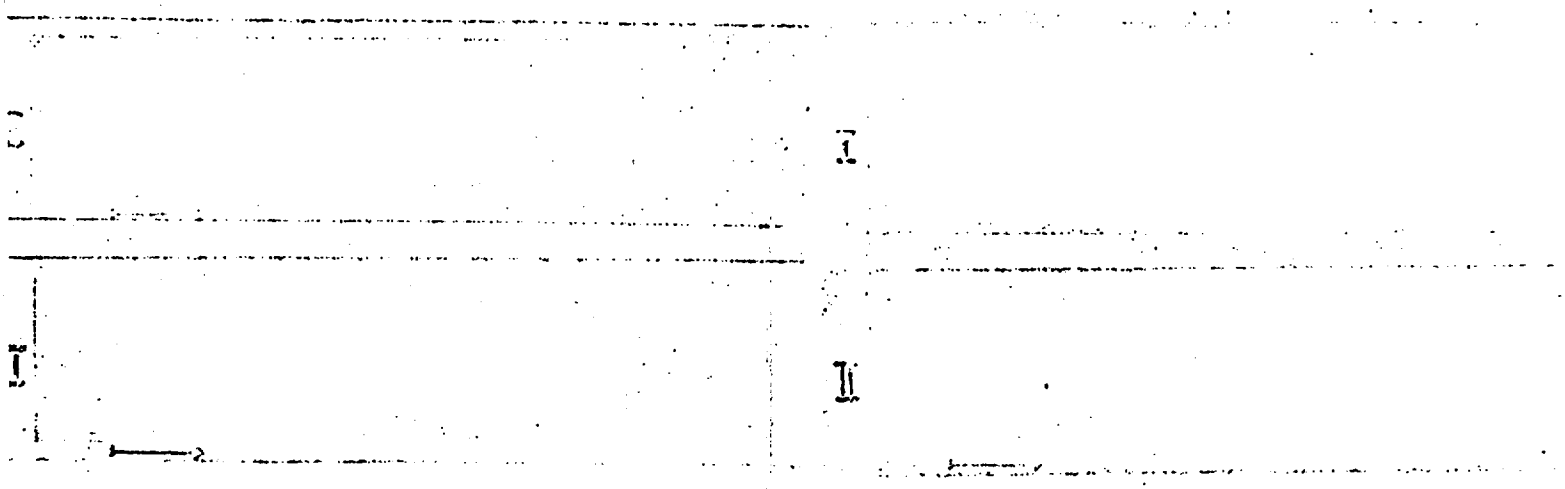


fig. 1a: electrophoresis on cellulose acetate foils

fig. 1b: paper electrophoresis

Fig. 1a: I=gelatin (from 1% gelatin solution, contains about 60 ugm gelatin, deposited with micro-stamp)
II= gelatin and milk egg white (isolated from 0.2% gelatin-content yoghurt, deposited with micro-stamp).
= direction of migration (anodic)
test duration: 3 hours; dyeing plus rinsing: 20 minutes

Fig. 1b: I= gelatin (from 1% gelatin solution, micropipette deposition of 10 ul, contains 100 ugm gelatin)
II= gelatin and milk egg white (isolated from a 0.2%-content-of-gelatin yoghurt, deposited with stamp).
= direction of migration (anodic)
Test duration: 14 hours;
Dyeing and rinsing time: 2 hours

The pherograms show that yoghurt 1 is free of gelatin and that there is gelatin in yoghurt 2. During gelatin isolation, the milk egg white in yoghurt 2 was completely precipitated by means of heat treatment and removed by means of centrifugal action or of filtration. For gelatin concentration, the filtrate will be raised to 10 fold concentration by vacuum evaporation. If the milk egg white is not completely removed, its egg white bands will appear on the pherogram (see picture of self-made yoghurt (b) and that for yoghurt 1). The pherogram shows remaining egg white still capable of migration in yoghurt 1. For the self-made yoghurt sample, -- yoghurt + gelatin --, one may observe completely denatured milk egg white (remaining at the place of deposition) and capable of migrating.

The pherograms therefore show that gelatine tests in dairy products do not require absolute purity in the separated gelatin Even if the extract obtained after yoghurt heat treatment and after gelatin concentration does contain some milk egg white, clean separation of egg whites from gelatin by means of electrophoresis is quite possible.

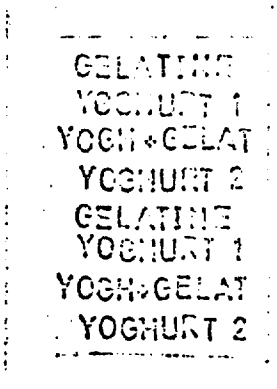


fig. 2

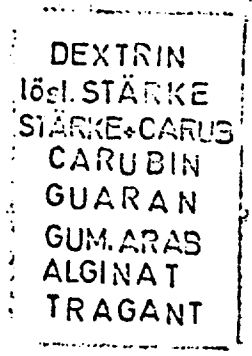


fig. 3
pherogram for PAS positive gels and thickening means.

Fig. 2: shows 8 pherograms from double samples deposited by means of micro-stamp

a) pure gelatin solution as comparison sample; 0.25 ul of 1% solution, corresponding to 2-3 ug of gelatin, deposited
b) extract from self-made yoghurt containing 0.2% gelatin, 10-fold concentration, 0.25 ul deposited, corresonding to 4-6 ug gelatin
c) extracts from commercially obtained yoghurt samples (yoghurt 1 and yoghurt 2); same concentration as in b).

TEST CONDITIONS: 200 volts; carbonate buffer of pH 10 and 0.075 micron ion size; anodic migration; separation time of 25 minutes; amido black dyeing (10 B).

Fig. 3: the amounts deposited are 4-5 ugm for dextrin; 2-3 ugm for soluble starch; 7-8 ugm for carubin; 7-8 ugm for guaran; 7-8 ugm for gum arabic; 2-3 ugm for alginate and 2-3 ugm for tragacanth.

TEST CONDITIONS: 200 volts; borate buffer of Ph 10 and ion size of 0.065 microns; anodic migration; separation time of 15 minutes; PAS dyeing.

2.4.3 MICRO-ELECTROPHORESIS OF GELS AND THICKENING MEANS OF THE POLYSACCHARIDE GROUP

We selected borate buffers with a pH of 10 and ion size of 0.065 microns as the conducting electrolytic solution for the electrophoresis of gels and thickening means of the polysaccharide group. Aside from dyeing, the other test conditions are about the same as those for gelatin (section 2.4.2). Section 4 describes execution of dyeing and of micro-electrophoresis. Fig. 3 shows the pherograms of PAS positive gels and thickening means, fig. 5 shows those made visible following tannin preparation and PAS dyeing, and fig. 6 shows those dyed with toluidine blue O. The pherograms of fig. 4 show the results of electrophoresis separation for mixtures from 2 to 4 different PAS positive gels and thickening means. Section 3 will discuss in detail the dye-affinity of the individual gels and thickening means and their classification according to the dyeing method used.

The pherograms show not only that the gels and thic kening means tested migrate at different rates, but also that they are sensitive in different degrees to dyeing.

For the test conditions as selected, the alginate and the tragacanth spots are subject to a remarkable though constant curvature (see also fig. 5 and 6) the cause of which is unknown to us. For micro-electrophoresis with borate buffer, gelatin too evidences this effect (fig. 5), but this is not the case when carbonate buffers are used (fig. 2).

In the pherograms shown in fig. 3, carubin, guaran and gum arabic evidence nearly the same migration rate. If a longer separation time is used, namely 25 minutes, -fig. 4 -, in lieu of 15 minutes, they may be separated from one another.

If the pherograms of figures 5 and 6 are more closely examined, it will be observed that several gels and thickening means migrate nearly at the same rate. Agar- [illegible]r, carubin and tragacanth in fig. 5, carrageen, sodium pectate and [illegible]inate in fig. 6 therefore cannot be separated from one another [illegible]eans of electrophoresis under the given test conditions. In ord[illegible] identify them, an improved separation may be achieved as [illegible]he case of the PAS positive gels and thickening means (fig. 4) [illegible] longer separation time of 20-30 minutes, and they may be furth[illegible]differentiated because of their variable dye-ability (see table [illegible],section 3).

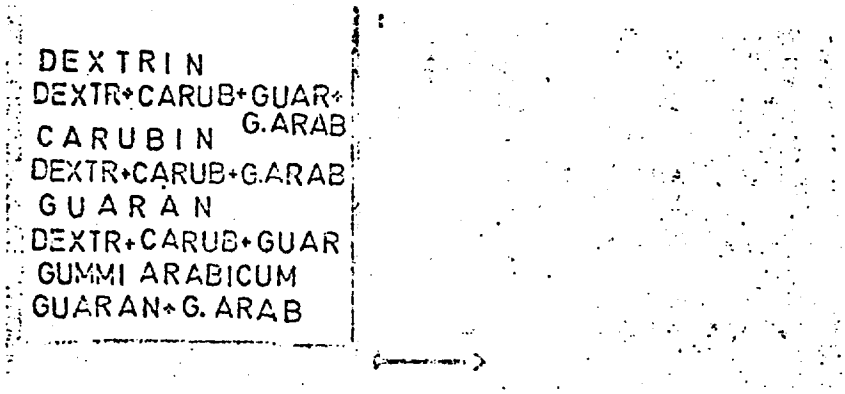


Figure 4

Electrophoresis-separation of mixtures of PAS positive gels and thickening means.

Deposited amount for dextrin was somewhat less (2-3 micrograms in lieu of 4-5), amounts for carubin and guaran were somewhat more (9-10 microgms in lieu of 7-8) than was the case for pherograms in fig. 3. Longer test duration was required for their separation (25 minutes in lieu of 15). Test conditions:

terminal potential: 200 volts
borate buffer: pH = 10; ion size: 0.065 microns
anodic migration
separation time: 25 minutes
PAS dyeing

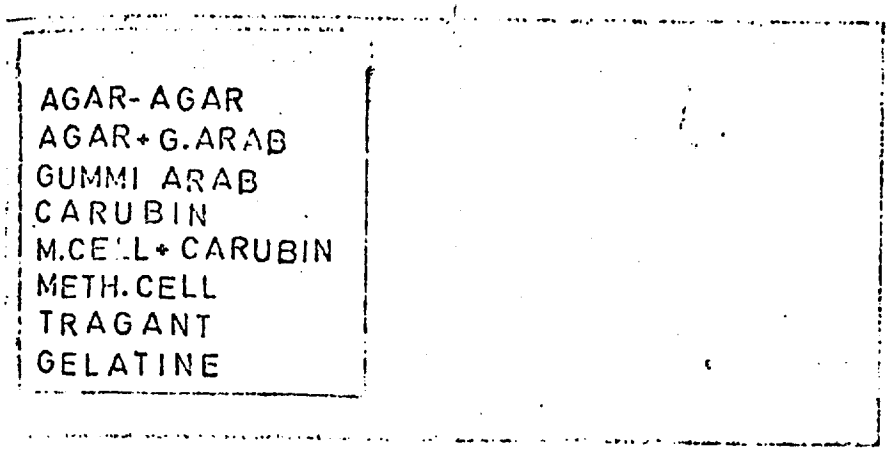


Fig. 5
Pherograms of gels and thickening means rendered visible by means of tannin and PAS dyes preparation.

The deposited amount for agar-agar is 4-5 microgm, for gum arabic 7-8 microgm, for carubin 8-10 microgm, for methyl cellulose 4-5 microgm, for tragacanth 4-5 microgm, and for gelatin 2- microgm.
Test conditions:

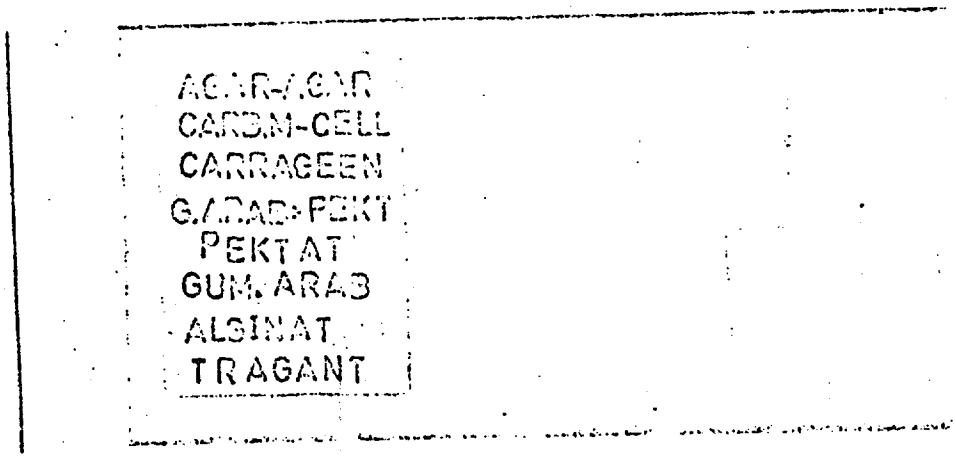
terminal potential: 200 volts
borate buffer pH: 10; ion size: 0.065 microns
anodic migration
separation time: 15 minutes
tannin and PAS dyeing preparation*

SUMMARY

The analysis process described enables identification of individual gels and thickening means as such. In the OFFICIAL METHODS OF ANALYSIS of the AOAC (1965) is listed a method allowing separation of gels and thickening means in food products such as ice cream and mayonnaise after removal of fat and egg white components by means of alcohol precipitation. If this precipitation reaction is positive, then electrophoresis and our dyeing method will allow precise identification of the alcohol precipitate.

Quantitative evaluation may take place by comparing with the color intensities of solutions of known concentrations of the particular gels and thickening means. Coarse estimates may be made by glance alone, photometry being appropriate for precise measurements.

Fig. 6
Pherograms of gels and thickening means with affinity for toluidine blue O



The amounts deposited are, for agar-agar, 4-5 ugm; for carboxy methyl cellulose, 4-5 ugm; for caarageen, 2-3 ugm; for pectate, 4-5 ugm; for gum arabic, 7-8 ugm; for alginate, 2-3 ugm; and for tragacanth, 4-5 ugm.

Test conditions:
- terminal potential: 200 volts
- borate buffer pH: 10; ion size: 0.065 microns.

anodic migration
- separation time: 15 minutes
- dyeing: toluidine blue O

3. DYEING OF GELS AND THICKENING MEANS ON CELLULOSE ACETATE FOILS

3.1 FIXING

Prior to dyeing, the gels and thickening means deposited on the cellulose acetate foils should be first fixed so as not to be dissolved during the dyeing process in the dyeing bath. As other egg white bodies, gelatin may be denatured by drying between 80 and 100°C and therefore be fixed, but this is not recommended for cellulose acetate foils, unfortunately, because of the wear on the foil. We make use of a fixing bath instead of the drying process, such bath consisting of a solution of trichloracetic acid for gelatin and of ethanol for the gels and thickening means of the polysaccharide group.

3.2 GELATIN DYEING

Dyeing of gelatin, which belongs to the protein group, occurs with the conventional egg white substance amido black 10 B, an acid azo-dye. The dyeing process is based on salt formation of the dye's acid groups with the free groups from the egg white. For gels and thickening means from the polysaccharide groups, dyeing must be undertaken in another manner, because there are no free amino groups and therefore do not respond to amido black 10 B (see fig. 7 and table 1).

3.3 PAS DYEING

The PAS (PERIODIC ACID SCHIFF) reaction, recommended by HOTCHKISS (1948) for the dyeing of polysaccharides in animal and plant tissue preparations used in histology, was carried over on our part to gels and thickening means. It was found that not all gels and thickening means could be dyed in this manner. Therefore we make a distinction between PAS positive and PAS negative gels and thickening means.

The Schiff reagency used in PAS dyeing consists of a colorless solution of fuchsin-sulfur acid prepared from adding potassium pyrosulfite and hydrochloric acid to a red, aqueous fuchsin solution. The released sulfur dioxide reduces the red fuchsin to a colorless leuko compound. This Schiff reagency is a known reagency test for aldehyde groups. The gels and thickening means to be dyed first are allowed to react with periodic acid for the generation of aldehyde groups. Two neighboring hydroxyl groups in the polysaccharide molecule will be oxidized to aldehyde groups in the presence of C-C fission. These aldehyde groups react with the Schiff reagency and generate a lilac -red color. Carubin, guaran, soluble starch, dextrin, alginate, tragacanth and gum arabic belong to the PAS positive gels and thickening means. Sodium pectate and carrageen are slightly PAS positive, carrageen's color being very slow to appear.

Polysaccharides, methyl cellulose, cellulose acetates and agar-agar, that lack neighboring hydroxyl groups, correspondingly are PAS negative. Water soluble methyl cellulose shows an average substitution index of 1.64 - 1.92 (GLICKSMAN, 1963) and also contains methyloxyl groups not only as regards the carbon atom C-6 but also to some extent the carbon atoms C-2 and C-3.

For negative PAS and slightly positive PAS gels and thickening means, we found two other ways of dyeing:

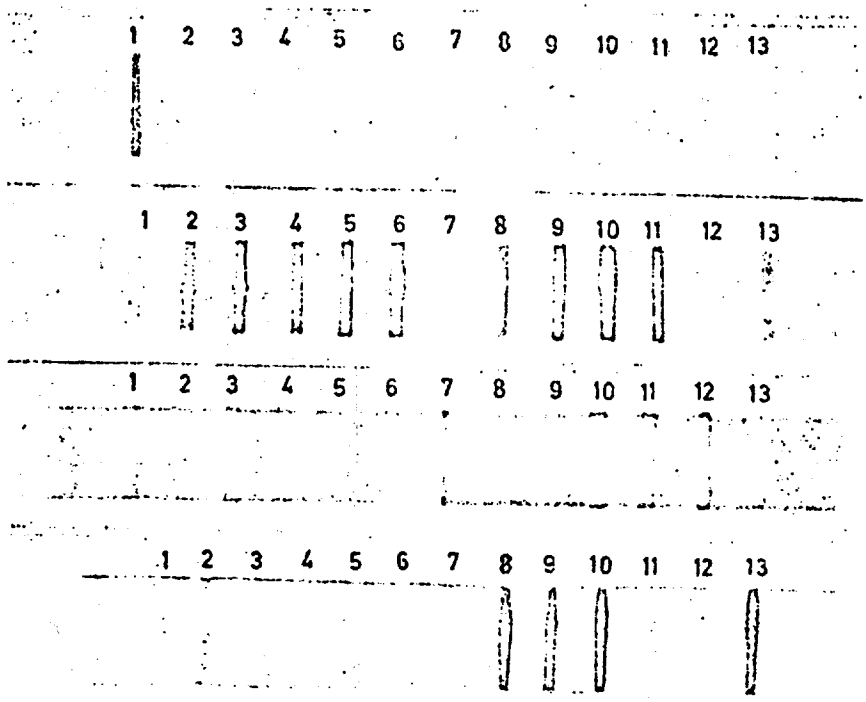
1. a prior treatment with tannin and subsequent PAS dyeing,
2. dyeing with toluidine blue O.

3.4 TANNIN TREATMENT AND PAS DYEING

We first considered using tannin for fixing the gels and thickening means. It was then shown that following such treatment, methyl cellulose could be dyed with the PAS reaction. Previously we had spent much time in vain looking for a way of dyeing methyl cellulose. Besides the latter, gelatin, carubin, agar-agar, tragacanth and gum arabic may also be rendered visible in this fashion. The tannin reaction occurring during this dyeing process -- if so -- is not known to us, except for the corrosive effect.

Besides tannin, we tested many other precipitation means for the polysaccharides, such as lead acetate, phosphor tungsten acid, mercuric chloride and barium chloride, though without satisfactory results obtaining. It was solely when pre-treating with barium chloride that better dyeing of carboxymethylcellulose was achieved. However toluidine blue O provides more sensitive dyeing.

Fig. 7
Dyeing of gels and thickening means on cellulose acetate foils



Numbers 1 through 13 refer to the gels and thickening means in table 1 below. In this dyeing test, the samples were deposited by means of a stamp and about 1.5 cm apart on a membrane supported from a frame; membrane size was 2.5 x 30 cm. Each amount deposited contains about 6 ul of a 1% solution, that is, about 60 ugm of the particular gel or thickening means. Execution of dyeing is described in section 4.4.

3.5 DYEING WITH TOLUIDINE BLUE O

Toluidine blue O is a thiazine dye used in histochemistry for dyeing acid mucopolysaccharides such as heparin and chondroitin sulphates (RIENITS, 1953). Polysaccharide affinity for toluidine Blue O assumes the presence of acid groups in the molecule. Those gels and thickening means that may be dyed with toluidine blue O, that is, sodium pectate, alginate, tragacanth, gum arabic, carboxymethylcellulose and carrageen, contain either uronic acids or sulfate groups in the molecule.

3.6 RESULTS

By making use of the four different dyeing processes, all gels and thickening means admitted under article 443 bis of the Swiss Food Regulations may be dyed differently on cellulose acetate foils (fig. 7 and table 1).

Table 1
Affinity of gels and thickening means on cellulose acetate foils

Gelier- und Verdickungsmittel	Amido-Schwarz 10B	PAS-Anfärbung	Tannin-behandlung + PAS-Anfärbung	Tolidin-Blau O.
1. Gelatine	++	—	(+)	—
2. Na-Pektat	—	(+)	—	(+)
3. Carubin* ××	—	+	—	—
4. Guaran* ××	—	+	—	—
5. Lösliche Stärke	—	+	—	—
6. Dextrin	—	+	—	—
7. Agar-Agar	—	—		—
8. Carrageen	+		(+)	+
9. Alginat	—	+	—	+
10. Tragant	—	+	+	+
11. Gummi Arabicum	—		+	—
12. Methyl-Cellulose	—	+		—
13. Carboxy-Methyl-Cellulose	—	(+)	+	

++ high affinity
+ good affinity
(+) slight affinity
- no affinity

From the dyeing results for the gels and thickening means tested and listed in fig. 7, a listing is made in table 1 where the dyeing methods of the four different kinds used are referred to by plus or minus signs for easier visualisation.

4. PROCEDURE

4.1 SPECIAL EQUIPMENT

BECKMAN-SPINCO microzone electrophoresis system***, consisting of:

microelectrophoresis cell
micro-sample depositing stamp (0.25 ul sample)
cellulose acetate foil (also designated as membrane), 5.5 x 14 cm for 8 samples at a time
power supply (potential: 0-500 volts, 0-50 ma)

FOR ELECTROPHORESIS ON LARGE CELLULOSE ACETATE FOILS:

Elphor-H electrophoresis chamber of GRASSMANN & HANNIG, with associated rectifier and regulating transformer (Bender & Hobein, Munich, Zurich)
membranes of 4 x 30 cm (Schleicher & Schuell, AG, Feldmeilen)
sample depositing-stamp, about 6 ul sample (Kontron AG, Zurich)

REAGENTS:

borate buffer (pH=10, ion size = 0.13 microns): 12.37 gm (= 0.2 mol) boric acid in 100 ml 1-n NaOH solution, completed with distilled water to 1 liter. Then mix 600 ml of this solution with 400 ml of 0.1-m NaOH.

sodium carbonate, sodium hydrogen carbonate buffer (pH=10, ion size = 0.15 microns): mix 750 ml 1-m sodium carbonate solution with 750 ml of 0.1-m sodium hydrogen carbonate solution and with 500 ml distilled water.

PERIODIC ACID SOLUTION: 2 gm periodic acid in 10 ml distilled water solution, then mix with 90 ml 96% vol. ethanol.

TRICHLORACETIC ACID: 5% aqueous solution

TANNIN SOLUTION: 10% aqueous solution ****

SATURATED AMIDO BLACK 10 B SOLUTION: about 0.1 gm amido black in a mixture of 9 parts volume methanol and 1 part volume glacial acetic acid, dissolution through repeated shaking. Solution must be filtered prior to use.

SCHIFF's REAGENT: 1 gm fuchsin in 100 ml distilled water, hot dissolution, cooling to about 50°C; mixing with 1 ml concentrated hydrochloric acid and 2 gm potassium pyrosulfite, hard shaking, rest over night. Shaking with about 1gm charcoal prior to use and filtering.

TOLUIDIN O SOLUTION: 0.2% aqueous solution

formaldehyde-AMMONIA MIXTURE: mix 1-m formaldehide solution with 1-m amoonia hydroxide solution in equal parts volume.

ALCOHOL-HYDROCHLORIC ACID MIXTURE: mix 1 part volume 1-n hydrochloric acid with 2 parts volume denatured alcohol (aceton spirit).

METHANOL - GLACIAL ACETIC ACID MIXTURE: 1 part volume glacial acetic acid with 9 parts volume methanol

TRANSPARENCY SOLUTION: 1 part volume glacial acetic acid and 3 parts volume methanol (always fresh).

methanol-SOLUTIONS OF TESTED GELS AND THICKENING MEANS: 1% solution of individual gels and thickening means in the borate buffer. Exception: a concentration of only 0.3% for agar-agar, because a 1% solution may already cause gel binding. As regards carubin, guaran and tragacanth, only about 20% of the substance in borate buffer goes into solution.

4.3 EXECUTION OF MICRO-ELECTROPHORESIS

4.3.1 FILLING THE MICRO-ELECTROPHORESIS CELL WITH BUFFER SOLUTION

The micro-electrophoresis cell consists of the buffer containers, of the electrode chambers, of a support frame for tensioning and a membrane, of a cell-cover and a cell upper part with slits and grooves for the micro-depositing stamp when depositing samples. Upon removing the cell cover, the cell upper part and the support frame, the siphon located between the electrode chambers will be made horizontal by means of one finger and the cell will be filled through the siphon opening to a height between the lines FLUID LEVEL with buffer solution. When testing gels and thickening means of the polysaccharide group, borate buffer with a pH of 10 and ion size of 0.065 microns is used, and for the gelatin test, a buffer of sodium carbonate / sodium hydrogen carbonate with a pH of 10 and ion size of 0.075 microns. The buffer solutions described in section 4.2 to that end will be first diluted with distilled water in equal parts volume. Following filling of the micro-electrophoresis cell, buffer drops on the cell wall above the buffer level are carefully removed by means of filter paper in order to avoid secondary contacts of electrical current beyond the membrane.

4.3.2 EMPLACING THE MEMBRANE

The membrane first is slightly wetted with the non-diluted buffer being used (see section 4.2). In order to wet evenly, the membrane is placed flat on the surface of the buffer solution. After wetting, the foil is dipped into the buffer solution by means of tweezers. White air inclusion spots are thus avoided. The wet membrane then is removed and easily compressed between two sheets of thick filter paper.

Next the evenly wetted membrane is so tensioned in the supporting frame that all the pegs of the latter fit into the membrane's holes, and therefore the membrane will be held equally tautly everywhere. The supporting frame with tensioned membrane will be so inserted in the micro-electrophoresis cell that the reference hole of the foil will precisely coincide with the numeral 1 of the numbers marked on the cell upper part. This aids remembering the sequence of the deposited samples.

4.3.3 SAMPLE DEPOSITION

After emplacing the cell upper part, deposition of samples may begin. By means of a glass rod, a drop of the solution to be tested is put on a glass plate that is kept neat. The micro-sample depositing stamp by means of its platinum laminae touches the sample drop and the liquid film so created is deposited on the membrane, the platinum laminae remaining about two seconds in touch with the membrane. Prior to the next sample deposition, the platinum laminae of the micro stamp will be rinsed with distilled water and dried by means of careful dabbing with filter paper. Once all eight samples have been deposited, the cell cover is put into position and electrophoresis migration may begin.

4.3.4 ELECTROPHORESIS MIGRATION

The micro-electrophoresis cell is so connected to the power supply that the location of sample deposition is on the side of the cathode. Sample migration is toward the anode. Terminal potential is set to 200 volts. For the buffer concentration used, the current density does not exceed 1 ma/cm of foil width (= 5.5 ma), which is a good value for the migration and separation of the samples that were tested. Upon termination of the desired test duration, which may range from 15 to 30 minutes, the current is shut off, the electrophoresis cell is opened, the membrane is removed and then dyed. Dyeing takes place as described in section 4.4.

4.4 DYEING OF GELS AND THICKENING MEANS

The four different dyeing methods will be discussed in the sections below.

4.4.1 AMIDO BLACK 10 B DYEING

This method is used for rendering visible gelatin besides other egg white bodies. In order to fix the egg whites, the membranes will be immersed for 2-3 minutes in 5% trichloracetic acid solution. Then dyeing takes place for 10 minutes in the saturated amido black 10 B solution. Bleaching of that part of the membrane not carrying egg white takes place when the foil is immersed in a mixture of methanol and glacial acetic acid. The solution will be decanted after 2-3 minutes and replaced by a fresh one. Following threefold washing, white foils are obtained, on which gelatin spots and possibly others appear dyed deep-blue. The foil is rendered transparent in accordance with section 4.5.

4.4.2 PAS DYEING

This method is used to render visible carubin, guaran, soluble starch, dextrin, alginate, tragacanth and gum arabic. The membrane is inserted for 5 minutes in a periodic acid solution. This results in oxidation and also simultaneously in fixing by means of the alcohol of the periodic acid solution. Thereafter the membrane is immersed for 10 minutes in Schiff's reagent so that the individual spots of the tested gels and thickening means appear dyed lilac-reddish. In order to remove the excess Schiff reagent, the membrane is placed for one minute in a mixture of formaldehyde and ammonium, so that solution and membrane become intensely red because of the released fuchsin. In order to bleach that part of the foil free of gels and thickening means, the foil will be rinsed first with denatured alcohol and then several times with the mixture of alcohol and hydrochloric acid. The colortone of gels' and thickening means' spots changes from lilac-red to red-violet. The foil is rendered transparent in accordance with section 4.5

4.4.3 PRE-TREATMENT WITH TANNIN AND PAS-DYEING

This method is mostly used for rendering visible methyl celllulose and agar-agar. Other gels and thickening means that may also be dyed in this manner are gelatin, carubin, tragacanth and gum arabic. The membrane is placed for 7-8 minutes in the 10% tannin solution and then for 5 minutes in the periodic acid solution. Upon periodic acid treatment, foil and solution turn brown. The brown solution is decanted and replaced by new periodic acid. Dyeing proceeds as described in section 4.4.2

4.4.4 TOLUIDINE BLUE O DYEING

This method is mostly used for rendering visible carrageen, carboxymethylcellulose and sodium pectate. Other gels and thickening means that may be dyed with toluidine blue O are alginate, tragacanth and gum arabic. The membrane is placed for 10 minutes in the dye solution. Then the major part of the excess dye is removed by compressing the dyed foil between two sheets of filter paper. Following rinsing with ordinary tap water, the membrane is air dried.

4.5 RENDERING FOILS TRANSPARENT FOR PHOTOMETRIC EVALUATION

Following dyeing, the membrane is immersed for 2-3 minutes in methanol. Then it is placed on a glass plate or disc and subjected for 30 seconds to a fresh mixture of 3 parts volume methanol and 1 part volume glacial acetic acid. Glass plate and membrane are removed from the methanol / glacial acetic acid bath. Upon removing the excess liquid with a rubber sponge or filter paper, they are dried at 110°C for five minutes in an oven. The now transparent pherograms may be kept on the glass plates. They may be carefully removed from the glass plates for the purpose of photometric evaluation and they may be placed in glassines or the likes. The foils treated with toluidine blue O are not subjected to the methanol / glacial acetic acid bath because the spots of the tested gels and thickening means would dissolve, except for carrageen.

Gratitude is expressed to Herr Wenger, chemist from Bern, for verification of this work.

* Only a part hardly visible in this reproduction of the deposited agar-agar migrates on the cellulose acetate foil because of electrophoresis. The component initially remaining is more visible. If dissolved in 0.1-n hydrochloric acid and subsequently neutralized, agar-agar lends itself to easier deposition and will be rendered more easily visible than when in a buffer solution. It was also further shown that for pre-treating agar-agar with tannin, a 0.1% alcohol tannin solution is better suited than the reviously used 10% aqueous tannin solution.

** Carubin and guaran dissolve only to 16-19% in borate buffer. The insoluble part was subjected to centrifugation. Depending on quality, dye affinity of carubin will be more or less pronounced. It is strongest for carboxymethyl-carubin; it is very slight for carubin-fleur. Affinity as a function of quality and origin is being tested for further commercial products.

*** Representative: Kontron AG, Zurich

**** A 0.1% alcohol solution gave better results in more recent tests.

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The Effect of Various Adjuvants to the Diet of Rats on the Changes in Body Fats Induced by Feeding Soybean Oil[1]

By J. A. Schulz and B. H. Thomas

The early researches of Ellis, Hankins and associates and their extensive cooperative investigations on soft pork production (10, 13, 14) definitely established the fact that swine tend to transfer to body depots the type of fat fed them. Many other investigations in this and other countries have shown that when swine are fed more than minimal amounts of "softening" fats the body fat of the hog will be softer than usual, when considered practically, unless the feeding of such fats is discontinued at an early age and a more "hardening" feed mixture is substituted (5, 15, 16, 26).

Anderson and Mendel (1) checked some of the work of Ellis and associates. They used rats as the experimental animals and found that rats tend to deposit fats in much the same manner as do fattening hogs. They found, as did Ellis and Hankins with hogs, that as the rats grew the fats deposited became harder, and that when certain vegetable oils, such as soybean, corn and peanut oils were fed the fats deposited became softer and had higher iodine numbers. They further found that when rats fed thus were fasted until they had lost approximately one-fourth of their body weights, and then for a period of time were fed a diet having a smaller percentage of fat, the fats found subsequently in the body were harder and had lower iodine values. The above authors have also published other papers (18a, 19a) in which investigations they utilized the rat as a satisfactory subject for the study of fat metabolism.

Sinclair (17, 22) stated that the iodine numbers of the neutral deposited fats tend to rise in proportion to the iodine numbers of the fats fed but not strictly so. Also some of the work of Barbour (2, 3) and Chaikoff (7) should be considered. Robinson, Gray and Newton (20) fed to rats diets modeled after typical swine rations (but so modified as to be suitable for rats) in which were included 5, 10 and 20 percent of ground soybeans. They found that the iodine numbers of the body fats of the rats were considerably higher than those of the body fats of pigs fed similar rations.

Schoenheimer and Rittenberg (21) in their brilliant series of researches have proved, by incorporating deuterium as an indicator

[1]Project 507 of the Iowa Agricultural Experiment Station.

Iowa Agr. Expt. Sta, Bull. No. 336, 1945

into the fats fed to mice, that deposited fat is not the relatively inert material it was once regarded. Their data showed that in both the liver and the depots, fat is undergoing constant and rapid changes. These authors proved beyond a doubt the thesis long held, that desaturation of the fatty acids in the liver was the first step in the breakdown of the fatty acids. They also stated that while it was probably the more natural function of the liver to desaturate and break down long chain fatty acids into shorter ones, it was also possible for the organism, under certain conditions, to hydrogenate or saturate unsaturated fatty acids. Stetten and Schoenheimer (24) further reported that when palmitic acid was fed to rats, some of it was converted to stearic acid, thus lengthening the chain of carbon atoms by two.

The researches of Channon and associates (6, 9) present some new views to this problem, demonstrating that n-hexadecane was very efficiently absorbed from the alimentary tract of the rat and the cat, and partially deposited as such in the tissue lipids, where its presence decreased the iodine numbers of such lipids. Furthermore, this hydrocarbon was partially oxidized, presumably in the liver, to fatty acids. These observations have been confirmed recently by Stetten (34), who fed n-hexadecane containing deuterium. By appropriate analyses of the lipids, Stetten proved that this paraffin hydrocarbon can be absorbed from the alimentary tract of rats and that much of it may be oxidized to fatty acid in the body and used as such. Stetten (25) also has reported that the acetates of cetyl and octadecyl alcohols are readily absorbed by "normal" rats and rapidly converted to saturated fatty acids of the same carbon chain length.

Frazer (11) found that fats are absorbed from the intestinal tract both as fats and as fatty acids and that fats as such traveled largely through the lacteal lymphatic route, short circuited the liver and thus passed more or less directly into fat depots. Frazer, Stewart and Schulman (12) found that finely emulsified liquid paraffin could be absorbed from the alimentary tract as well as neutral fats.

Nowhere in the extensive literature on fat metabolism, including that of the production and prevention of fatty livers in rats, have the authors found an absolute interdiction of the idea that it might be possible to feed to the animal substances which would in some way modify the normal fat metabolism so that fats of a firmer and more desirable consistency could be produced. Schoenheimer and coworkers (21, 24) have shown that modifications in normal fat metabolism take place naturally. Also, in the earlier work of Ellis (10), Anderson and Mendel (1), Sinclair (22), etc., whenever fats of high iodine value were fed, the animals deposited fats of a somewhat lower iodine value. When fats of a low iodine value, such as coconut oil or butter, were fed, the deposited fats had higher iodine values than those of the fats fed.

The work reported in this paper was initiated in the hope that some substance or "adjuvant" might be found which, when fed to rats receiving a diet containing a "softening" fat, would tend to modify the normal fat metabolism so that the body fat of such rats would be of firmer consistency than that of rats similarly fed but containing no such adjuvant in the diet.

The basal diet had a softening effect by virtue of the fact that it contained 10 percent soybean oil, and it was to this diet that the various substances chosen as adjuvants were added. An additional group of rats received no oil, so that fat deposited by them would be of maximum firmness. Another group of rats received soybean oil amounting to 30 percent of the diet to ascertain if the fat deposited by these rats would be notably softer than fat deposited by rats receiving the basal (10 percent soybean oil) diet without adjuvants.

Finally, it was considered that if these experiments with rats led to the discovery of adjuvants which would favor the deposition of firm body fats, under the conditions described, such adjuvants might be used in studies in which fattening hogs would be utilized as the experimental animals.

EXPERIMENTAL

GENERAL PROCEDURES

This study was conducted in two parts. In the first part numerous adjuvants were incorporated in the diet to determine whether any excelled for the purpose intended. Based on results obtained from the first trial, the second trial was limited to testing a few adjuvants which at the time seemed to offer the most promise.

In both tests the kind of rat, the method of feeding and management, and the rat-salvaging procedure used were essentially identical. The tests were conducted in a well-ventilated laboratory which was maintained at approximately the same temperature throughout the tests. Each test lot consisted of six or seven young rats of both sexes and ranged in weight from 60 to 80 grams when taken from the stock colony. In the second trial only male rats were used. Each rat was fed and cared for in an individual metal cage with ½ inch wire mesh bottom. All rats used in each trial were started on feed at approximately the same time. The rats used in the first trial were fasted for 48 hours before they received the experimental feed, but in the second trial the rats were fed the experimental diets as soon as they were placed on test. Each rat received his feed ad libitum from an approximately "non-spilling" feeding device, which consisted of a 2-ounce "vaseline" jar that contained the feed, placed within a widemouthed 8-ounce "mayon-

naise" jar, which was wired to the side of the cage. The rats balanced themselves on the edge of the outer jar while eating, but rarely carried food to the top of the outer jar. The small amount of feed they spilled was easily recovered from the paper towels which lined the trays over which the cages were suspended. The rats were weighed daily during the first week, then semi-weekly, and later, weekly.

All excepting three groups of rats, two of which were fed positive and one negative control rations, were fed a basal ration consisting of solvent extracted soybean oilmeal 50 percent, soybean oil 10 percent, salt mixture (table 1) 1.5 percent, yeast 2 percent or yeast concentrate 1 percent, and variable amounts of sugar or starch to make a total of 100 percent. The different adjuvants tested replaced equal weights of sugar or starch in the basal diet where more than 1 percent of the adjuvant was fed, otherwise the adjuvant was merely added to the basal ration (see table 1). Consequently in the 17 rations fed, the percentage of sugar or starch ranged from 0 to 46.5 in the case of the lactose and positive control rations, respectively. The adjuvants used were chosen because of their physiological oxidative or reductive properties, or because they had previously been linked experimentally at some time with fat metabolism. [2]Table 1 presents the diets fed.

The rats were kept on test until they ceased growth or until after they had reached a weight of approximately 250 grams. Consequently, the number of days the rats were on test varied from 54 to 110.

As each rat was removed from the experiment it was lightly anesthetized with illuminating gas, carefully decapitated and the blood saved by permitting it to flow into a Bailey Walker fat extraction flask which contained 35 ml. C_2H_5OH (95 percent), 5 ml. water, and 4 grams solid KOH. The liver of each rat was removed and placed into a flask similarly prepared. The intestinal tracts of the rats were then removed and the bodies of the rats minus liver, blood and intestinal tracts were frozen and stored under refrigeration at —20°C. The frozen bodies were later cut into small pieces and ground through a small meat grinder. Two or three tightly

[2]Specifically: l-cystine, dl-methionine, "choline chloride," niacin and casein were used as adjuvants because of their wide use in studies of fatty livers in rats. Casein was also used because the effect of additional protein in the ration might be of interest.

Carotene and ascorbic acid were used because of their reductive action in the body.

The calcium salts were used—one alkaline, one acid and one neutral because early work with children has indicated that the formation of calcium soaps in the intestinal tract influences fat absorption and excretion. Bile salts were also used because of their possible effect on the digestion of fat. Lactose was fed because it alters the flora of the intestinal tract.

Agar and charred alfalfa were used with the idea that additional "bulk or roughage" might affect the digestion of fats; in addition "charred substances" have been reported to have some influence on fat digestion.

Sodium fluoride, although toxic, was used because extensive studies on fluorine toxicosis in this laboratory had indicated that the body fat of rats receiving fluoride frequently appeared to be altered.

TABLE 1. ADJUVANTS FED AND AMOUNT OF EACH WHICH WAS ADDED TO, OR WHICH REPLACED SUGAR OR STARCH IN, THE BASAL DIETS.A

Experiment No. I

Lot no.	Adjuvant and amount in diet	Amount	Lot no.	Adjuvant and amount in diet	Amount
S 1†	Positive control	0 soybean oil	S 9†	Negative control	30% soybean oil
S 2†	Basal diet	.....	S 10	Agar	10%
S 3	Charred alfalfa meal	20%	S 11	Casein	20%
S 4	l-cystine	0.5%	S 12	(C) Carotene	(per rat) .0140 gm. per week
S 5	dl-methionine	0.5%	S 13	(D) Calcium carbonate	2% or 1%
S 6	Ascorbic acid	(per rat) 25 mgs. per day	S 14	(D) Calcium chloride	2.2% or 1.1%
S 7	(B) Bile salts	0.5%	S 15	(D) Calcium lactate	6.2% or 3.1%
S 8	Sodium fluoride	.05%	S 16	Lactose replaced all sucrose	36.5%

Experiment No. II

Lot no.	Adjuvant and amount in diet	Amount	Lot no.	Adjuvant and amount in diet	Amount
S 17†	Basal diet	.....	S 20	(E) Carotene	.0070 } gm. per rat per week
S 18(G)	Choline chloride (Merck)	1%	S 21	(E) Carotene	.0140 }
S 19	Niacin (nicotinic acid)	1%	S 22	(E) Carotene	.0280 }

(A) Basal diets	Expt No. I	Expt No. II
*Soybean oilmeal	50%	50%
Soybean oil	10%	10%
**Salt mixture	1.5%	1.5%
Yeast	2%	0
Yeast concentrate	.....	1.0%
Cane sugar	36.5%	37.5%
Starch	....	....
Total	100%	100%

Each rat received weekly three drops of codliver oil concentrate.

*Soybean oilmeal = solvent extracted meal containing 0.5% fat.

**Salt mixture S1 fed all lots of rats except Lot S14=$CaCO_3$ 55, bonemeal 20, NaCl 20, ferric citrate 4.5, KI 0.05.

Salt mixture S2 fed Lot S14=$CaCl_2$ 55, $Ca_3(PO_4)_2$ 20, NaCl 20, ferric citrate 4.5, KI 0.05.

$CaCl_2$ replaced $CaCO_3$ in this salt mixture since acid conditions in the rats' intestinal tract were desired (4).

†These diets contained no adjuvant.

(B) Bile salts="Difco special for McConkey's agar."

(C) Carotene trial 1. 1 gm. carotene was dispersed in 36 gms. coconut oil to which 7 mg. pyrogallol had been added as an antioxidant. The carotene suspension was kept in a solid state in the refrigerator and each rat received 0.5 gm. of the solidified oil, bearing .014 gm. carotene per week.

(D) Rats in lots S13, S14 and S15 were not eating or growing well after one month on test; therefore, the percentages of calcium salts in the diets fed as adjuvants were halved. Feed consumption and growth were then improved.

(E) Carotene trial No. 2. Dispersed in soybean oil.

(G) Choline chloride (Merck) is probably the hydrochloride, but is referred to as choline chloride throughout the text.

folded paper towels followed the body of each rat through the grinder and served to recover the last portions of the rat adhering to the machine. The towels did not become badly macerated since they were not forced through the grinder.

As each rat was ground, it was placed into a 750 ml. pyrex Erlenmeyer flask, together with 400 ml. water. A small beaker was inverted over the mouth of the flask and the resulting suspension was autoclaved at 20-25 lbs. for 15-20 hours. While still hot the liquid was decanted through a Buchner funnel into another 750 ml. flask, the meat residue was washed several times with boiling water and this liquid was also decanted through the funnel into the same flask. The aqueous solution was then separated from the fat by means of a separatory funnel. After the fat had been washed several times with hot water it was allowed to run into a 50 ml. Erlenmeyer flask with the aid of a little diethyl ether. The separatory funnel was washed free from adhering fat with diethyl ether, and the ether solution of fat was separated from the water and detritus. After the original flasks and aqueous solutions were again washed several times with small amounts of ether, the combined ether solutions were evaporated to small volumes and thoroughly dried with sodium sulfate. The water free ethereal solutions of fat were then filtered from the sodium sulfate; and the flasks, sodium sulfate and filter papers were washed with small amounts of anhydrous ether. After a preliminary evaporation of the ether solutions, either at a low heat or at room temperature, the flasks or vials were placed in a vacuum desiccator, and the remaining ether was removed by a continuous evacuation at room temperature. After all the ether was removed (24-48 hours) carbon dioxide was introduced into the desiccator, and the fats were stored under an atmosphere of carbon dioxide in the freezing compartment of a refrigerator until wanted for analysis. The sizes of the flasks and vials used for the storage of the fats varied with the volumes of the fats, to further minimize oxidation by air replacing the carbon dioxide, if the carbon dioxide escaped from the flasks.

The blood and liver of each rat were saponified separately overnight in Bailey Walker flasks over the low heat of electric hot plates. The solutions were then evaporated to small volumes to remove the alcohol, water was added, and the resulting solutions were extracted with diethyl ether to remove the sterols and other nonsaponifiable fractions. These ether extracts, after being washed with water and dried with sodium sulfate, were stored in ether solution for analysis later.

The alkaline water solutions of the blood and livers were acidified with HCl, and the fatty acids were then extracted with diethyl ether. These ether extracts after being washed and dried were also stored in ether solution. Before analysis, the ether was removed from both liver and blood nonsaponifiable fractions, and from liver and blood fatty acids in the manner previously described for the fats.

Finally, the iodine numbers of the body fats of the rats and the fatty acids of the bloods and livers as well as those of some of the nonsaponifiable fractions ("nonsaps.") of the bloods and livers were determined by means of the Rosenmund-Kuhnhenn Method as modified by Mario Yasuda (27).

EXPERIMENT I

RESULTS

Table 2 presents the data obtained from each rat used in Experiment I, while in table 3 these data are summarized for each of the 16 lots of rats used.

An examination of these tables shows that the rats varied rather widely in their rates of gain, since most of the lots contained one or two individuals which gained weight exceptionally well whereas others grew subnormally. The best average weight increase was made by the rats in lot S 11 (20 percent casein); whereas, the rats in Lot S 2 (basal), those in Lot S 12 (carotene) and those in Lot S 9 (30 percent soybean oil) grew at approximately equal but slower rates. The poorest average growth was made by the rats in Lot S 3 (20 percent charred alfalfa). Because of the methods used in rendering the fats from the bodies of the rats, the figures shown which express the weights and percentages of body fats must be regarded as approximations. These figures are presented only to show the wide differences in the amounts of fat contained in the bodies of these rats.

The iodine numbers of the body fats of the rats varied considerably among rats in the same lot, but apparently no more so than is customary in work of this nature. The iodine numbers of the body fats of the rats in Lot S 1 (low fat) varied least, and the body fats of these rats had by far the lowest iodine values. The body fats of the rats in Lot S 5 (methionine) presented the highest iodine values, though not greatly in excess of the iodine numbers of the body fats of the rats in Lots S 9 (30 percent soybean oil) and S 2 (Basal Diet). The body fats of the rats in Lots S 4 (l-cystine) and S 6 (ascorbic acid) also presented rather uniformly high iodine values. With the exception of the fats from the rats in Lot S 1 (low fat), the body fats from the rats in Lot S 12 (carotene) presented the smallest iodine values. A statistical study of the variation between treatments indicated that of the 13 adjuvants fed, 5 of these—carotene, agar-agar, sodium fluoride, bile salts and calcium chloride—may have significantly influenced the deposition of fats having smaller iodine values.[3]

[3]While the mean iodine numbers of the fats of the rats in these five groups do not vary widely, the iodine numbers of the body fats of the rats in Lot S 12 (carotene) were *uniformly* low, a primary reason for the use of carotene in Experiment II.

TABLE 2. DATA PERTAINING TO LIVE WEIGHT AND FEED CONSUMPTION, BLOOD AND TISSUE RELATIONSHIPS, AND CERTAIN BLOOD AND TISSUE ANALYSES OF RATS FED VARIOUS ADJUVANTS. EXPERIMENT NO. I.

Lot no. Adjuvant rat no. and sex	No. days on test	Live wts. and feed consumption					Blood and tissue wt. relationships						Analysis of blood and tissues							
		Initial wt. **	Final wt.	Gain in wt.	Total feed eaten	Feed req. per gm. gain in wt.	Total weights			% of body wt.			Liver fatty acids	Blood fatty acids	Liver non saps	I_2 no. body fat	I_2 no. liver fatty acids	I_2 no. blood fatty acids	I_2 no. liver non saps	I_2 no. blood non saps
							Body fat	Liver	Blood	Body fat	Liver	Blood								
		Gms.	Gms.	Gms.	Gms.	Gms.	Gms.	Gms.	Gms.	%	%	%	%	%	%					
S 1 Low fat diet																				
1 ♀	89	51	180	129	882	6.9	12.3	6.7	4.5	6.8	3.8	2.5	3.6	0.7	0.2	66.9	85.4	25.5	48.3	11.2
2 ♂	87	58	177	119	883	7.4	3.0	10.4	5.0	1.7	5.8	2.8	2.9	0.6	0.2	65.3	112.5	57.2	46.9	9.1
3 ♀	98	47	195	146	982	6.7	9.1	8.1	5.4	4.7	4.2	2.8	3.2	0.6	0.1	64.7	97.8	67.3	22.6	18.8
76 ♂	82	48	224	176	1073	6.1	7.8	12.1	7.4	3.5	5.4	3.3	3.0	0.6	0.1	64.0	107.1	44.9	69.7	25.2
75 ♂	85	53	189	136	898	6.6	4.9	7.0	5.9	2.6	3.7	3.1	3.3	0.6	0.1	64.8	103.9	58.7	18.8	25.4
74 ♀	55	49	154	105	.....	5.8	7.1	Died	Died	.....	.....	.....	.....	.....	.....	64.1	113.1	.....	48.0	.....
S 2 Basal diet																				
4 ♀	98	50	177	127	832	6.5	11.6	7.2	5.7	6.7	4.1	3.2	2.7	0.6	0.3	109.1	101.6	52.5	78.8	34.1
5 ♀	98	55	168	113	792	7.0	7.4	8.2	4.8	4.4	4.9	2.9	2.6	0.5	0.3	106.4	105.5	55.2	84.3	41.7
6 ♂	82	55	87	32	.....	13.3	.....	5.7	3.2	.....	6.6	3.7	2.1	0.8	0.4	.....	135.8	30.8	72.4	11.8
6a ♂	69	54	224	170	654	3.9	6.0	11.7	5.7	2.7	5.2	2.5	2.2	0.6	0.2	105.2	156.2	56.1	68.3	46.3
73 ♂	68	54	286	232	807	3.5	15.3	11.1	7.9	5.4	3.9	2.8	2.6	0.4	0.3	105.2	142.4	77.3	49.0	50.5
80 ♂	82	51	263	212	811	3.8	10.7	12.0	9.1	4.1	4.6	3.5	2.7	0.4	0.1	110.1	155.6	76.6	85.2	54.5
79 ♀	99	51	196	145	923	6.4	8.8	10.3	5.8	4.5	5.3	3.0	3.0	0.5	0.3	108.9	156.2	72.7	71.1	54.6
S 3 Charred alfalfa																				
7 ♀	94	51	131	80	709	8.8	1.8	6.6	3.8	1.3	5.0	2.9	3.6	0.5	0.3	97.1	70.2*	54.9*	*Ether contaminated. Figures much too low.	
8 ♀	99	47	141	92	841	9.1	Lost	7.2	4.2	.....	5.1	3.0	3.3	0.5	0.4	.....	88.5*	25.9*		
9 ♂	94	59	206	147	940	6.4	4.5	9.2	6.2	2.2	3.0	4.5	3.5	0.4	0.3	109.2	94.6*	34.9*		
78 ♀	94	57	156	99	775	7.8	4.1	7.3	4.4	2.6	2.8	4.7	2.7	0.5	0.3	101.7	127.3*	36.5*		
77 ♂	94	54	199	145	1021	7.1	3.4	9.7	6.6	1.8	3.3	4.9	3.5	0.4	0.3	101.2	76.1*	29.4*		
84 ♂	95	54	197	143	933	6.5	4.4	10.0	6.6	2.3	3.4	5.1	3.6	0.3	0.3	96.8	89.6*	35.4*		
S 4 L-cystine																				
10 ♀	103	53	192	139	933	6.7	5.7	8.1	4.7	3.0	4.2	2.4	3.2	0.5	0.6	104.5	60.5*	15.2*	*Ether contaminated. Figures much too low.	
11 ♀	96	57	198	141	798	5.6	8.2	9.1	4.6	4.1	4.6	2.3	3.5	0.5	0.5	108.9	70.2*	15.9*		
12 ♂	99	52	250	198	1057	5.3	11.2	12.7	8.5	4.5	5.1	3.4	3.4	Lost	0.3	108.5	68.8*	24.8*		
83 ♀	101	59	195	136	904	6.6	7.0	9.3	4.7	3.6	4.8	2.4	3.6	0.5	0.4	101.7	68.5*	25.0*		
82 ♂	99	52	202	150	873	5.8	5.0	10.5	5.5	2.5	5.2	2.7	3.3	0.4	0.3	100.7	66.1*	15.8*		
81 ♂	66	51	259	208	687	3.3	13.2	13.7	8.3	5.1	5.3	3.2	2.7	0.4	0.2	110.1	92.3*	38.5*		

Lot no. Adjuvant rat no. and sex	No. days on test	Live wts. and feed consumption					Blood and tissue wt. relationships						Analysis of blood and tissues							
		Initial wt. **	Final wt.	Gain in wt.	Total feed eaten	Feed req. per gm. gain in wt.	Total weights			% of body wt.			Liver fatty acids	Blood fatty acids	Liver non saps	I_2 no. body fat	I_2 no. liver fatty acids	I_2 no. blood fatty acids	I_2 no. liver non saps	I_2 no. blood non saps
							Body fat	Liver	Blood	Body fat	Liver	Blood								
		Gms.	Gms.	Gms.	Gms.	Gms.	Gms.	Gms.	Gms.	%	%	%	%	%	%					
S 5 Methionine																				
13 ♂	91	55	201	146	898	6.2	5.0	10.8	9.4	2.5	5.4	4.7	3.1	0.3	0.2	108.7	114.9	45.8	81.3	63.8
14 ♀	96	51	169	118	800	6.7	5.4	8.7	4.6	3.2	5.1	2.7	3.3	0.6	0.1	109.0	120.9	53.6	77.8	59.3
15 ♀	96	47	191	154	840	5.5	10.5	8.0	5.7	5.5	4.2	3.0	3.2	0.4	0.1	111.4	147.9	58.2	78.6	65.0
88 ♀	102	47	200	151	856	5.7	9.3	7.2	5.5	4.7	3.6	2.7	3.2	0.4	0.3	114.6	116.0	83.2	89.7	68.5
87 ♂	89	53	227	174	895	5.2	6.4	11.1	5.8	2.8	4.9	2.6	3.1	0.4	0.1	112.1	122.8	85.6	90.4	82.5
86 ♂	94	48	207	159	903	5.7	8.0	10.8	7.0	3.9	5.2	3.4	3.1	0.4	0.2	107.5	124.9	85.5	75.1	76.7
S 6 Ascorbic acid																				
16 ♂	91	61	237	176	911	5.2	7.9	10.9	5.9	2.3	4.6	2.5	3.0	0.5	0.3	105.2	125.0	89.6	.....	.....
17 ♀	110	55	180	125	898	7.2	6.7	7.0	5.0	3.7	3.9	2.8	3.3	0.4	0.4	103.4	115.0	96.9	.....	.....
18 ♀	110	51	195	144	911	6.3	8.7	8.0	.....	4.5	4.1	.....	2.9	.....	0.4	114.2	105.4	56.4	.....	.....
85 ♂	105	57	227	170	1009	5.9	7.2	10.4	6.0	3.2	4.6	2.6	3.2	0.4	0.3	102.3	119.8	116.2	.....	.....
92 ♂	89	55	244	189	921	4.9	8.0	12.7	7.3	3.3	5.2	3.0	3.0	0.5	0.2	108.9	133.3	95.8	.....	.....
91 ♀	108	47	201	154	1058	6.8	13.9	6.9	5.4	6.9	3.4	2.7	3.3	0.5	0.5	105.9	101.8	64.4	.....	.....
S 7 Bile salts																				
19 ♂	91	58	193	135	833	6.1	1.7	8.2	6.3	0.9	4.2	3.3	3.0	0.3	0.5	104.9	132.1	83.4	.....	.....
20 ♂	118	62	214	184	1143	6.2	9.0	10.0	7.9	4.2	4.7	3.7	2.8	0.4	0.4	98.3	131.8	88.1	.....	.....
21 ♀	104	61	194	142	961	6.8	7.3	8.0	5.3	3.8	4.1	2.7	3.4	0.5	0.5	100.5	155.6	51.8	.....	.....
90 ♀	101	50	200	145	883	6.0	2.0	9.1	6.1	1.0	4.5	3.0	3.1	0.4	0.3	90.7	138.8	87.2	.....	.....
89 ♀	106	48	197	153	1027	6.7	7.3	7.3	5.6	3.7	3.7	2.8	3.4	.....	0.5	100.3	159.5	82.4	.....	.....
96 ♂	91	44	217	170	937	5.5	3.9	11.3	6.6	1.8	5.2	3.0	3.1	0.4	0.5	92.5	134.8	87.0	.....	.....
S 8 NaF																				
22 ♂	93	53	209	156	884	5.6	2.6	9.7	5.7	1.3	4.6	2.7	3.2	0.5	0.3	99.8	128.1	42.5	79.6	.....
23 ♀	96	51	161	110	755	6.9	2.8	7.0	4.8	1.7	4.3	3.0	3.5	0.4	0.3	82.9	118.2	76.8	55.6	.....
24 ♂	93	61	230	169	966	5.7	4.6	11.1	5.9	2.0	4.8	2.6	3.1	0.5	0.2	96.0	115.6	87.6	94.6	58.0
95 ♀	107	48	191	143	1032	7.2	5.3	9.1	5.6	2.8	4.8	2.9	2.7	0.5	0.3	104.6	149.9	92.4	55.4	64.1
94 ♀	101	55	207	152	910	6.0	8.8	9.6	6.4	4.3	4.6	3.1	3.2	0.6	0.3	97.8	114.8	33.5	66.7	43.0
93 ♂	75	55	265	210	995	4.7	11.0	12.3	6.9	4.2	4.6	2.6	2.6	0.5	0.1	98.4	157.9	85.7	84.0	74.9

**Weights represent those taken after rats had been denied feed for 48 hours.

TABLE 2—continued

Lot no. Adjuvant rat no. and sex	No. days on test	Live wts. and feed consumption: Initial wt. **	Final wt.	Gain in wt.	Total feed eaten	Feed req. per gm. gain in wt.	Blood and tissue wt. relationships: Total weights: Body fat	Liver	Blood	% of body wt.: Body fat	Liver	Blood	Analysis of blood and tissues: Liver fatty acids	Blood fatty acids	Liver non saps	I_2 no. body fat	I_2 no. liver fatty acids	I_2 no. blood fatty acids	I_2 no. liver non saps	I_2 no. blood non saps
		Gms.	Gms.	Gms.	Gms.	Gms.	Gms.	Gms.	Gms.	%	%	%	%	%	%					
S 9																				
30% Soy oil																				
25 ♂	107	49	247	198	908	4.6	19.3	9.4	7.7	7.8	3.8	3.1	6.2	0.5	0.5	105.4	115.4	89.4	70.7	38.6
26 ♀	101	49	176	127	743	5.8	9.1	7.5	4.9	5.2	4.3	2.8	4.6	0.9	0.4	106.9	125.8	45.0	70.9	Lost
27 ♂	61	65	253	188	616	3.3	10.4	12.8	9.6	4.1	5.1	3.8	3.5	0.4	0.1	108.0	178.8	95.8	68.8	48.8
52 ♀	89	51	181	130	741	5.7	7.8	8.3	5.0	4.3	4.6	2.8	5.5	0.7	0.4	106.4	133.9	41.2	76.0	23.6
51 ♀	101	54	205	151	826	5.5	17.6	7.4	6.2	8.6	3.6	3.0	5.2	0.8	0.3	110.7	134.9	39.0	73.5	26.8
50 ♂	75	53	246	210	788	3.7	10.7	11.8	5.8	4.4	4.8	2.4	3.2	0.6	0.2	110.5	150.0	77.8	78.1	34.2
S 10																				
Agar																				
28 ♂	107	52	238	186	1167	6.3	6.3	11.9	7.5	2.7	5.0	3.1	3.3	0.3	0.3	104.7	145.1	99.3	75.4	67.9
29 ♀	102	51	166	115	823	7.2	4.8	6.9	4.6	2.9	4.2	2.8	3.1	0.4	0.4	89.1	122.2	40.1	55.4	54.4
30 ♂	102	52	188	136	959	7.1	2.8	10.0	5.5	1.5	5.3	2.9	3.2	0.3	0.2	105.3	126.5	41.3	80.6	55.1
49 ♀	102	52	224	172	1123	6.5	4.1	12.3	7.2	1.8	5.5	3.2	3.2	0.4	0.3	89.4	125.9	98.6	75.1	55.1
56 ♀	89	58	192	134	836	6.2	8.0	7.8	4.8	4.2	4.1	2.5	3.3	0.4	0.3	97.2	126.7	45.1	69.1	63.0
55 ♂	82	46	209	163	870	5.3	5.2	11.1	6.0	2.5	5.3	2.9	3.2	0.5	0.2	91.3	133.6	90.6	71.7	64.2
S 11																				
Casein																				
31 ♀	93	51	224	173	922	5.3	11.3	8.6	6.7	5.0	3.8	3.0	2.8	0.4	0.2	96.9	126.4	61.8	.....	.....
32 ♂	61	54	301	247	778	3.1	10.2	16.1	9.1	3.4	3.5	3.0	2.4	0.4	0.1	96.2	147.8	87.9	.....	.....
33 ♀	93	54	217	163	912	5.6	9.3	8.0	6.8	4.3	3.7	3.1	3.0	0.5	0.3	103.1	143.4	94.6	.....	.....
54 ♀	96	54	215	159	993	6.2	14.8	8.6	5.7	6.9	4.0	2.6	3.1	0.5	0.6	98.7	140.6	45.3	.....	.....
53 ♂	54	45	288	243	687	2.8	10.6	15.5	8.5	3.7	5.4	2.9	2.6	0.3	0.3	96.9	181.4	85.8	.....	.....
60 ♂	54	49	280	231	658	2.8	13.5	13.5	8.3	4.8	4.8	3.0	2.2	0.4	0.2	106.0	150.7	61.7	.....	.....
S 12																				
Carotene																				
34 ♀	89	49	176	127	730	5.7	6.5	7.5	4.5	3.7	4.3	2.6	3.5	0.7	0.3	99.3	115.9	42.7	37.4	66.4
35 ♀	89	49	153	104	682	6.6	5.6	7.7	4.5	3.7	5.0	2.9	3.5	0.6	0.2	92.3	107.6	69.9	75.1	66.9
36 ♀	96	53	182	129	824	6.4	7.1	7.6	5.3	3.9	4.2	2.9	3.3	0.5	0.2	95.9	112.6	75.8	49.1	49.6
59 ♂	84	55	198	143	714	5.0	2.7	10.4	5.5	1.4	5.2	2.8	3.1	0.5	0.2	98.5	114.2	31.0	33.1	69.6
58 ♂	56	60	287	237	758	3.2	8.3	14.2	9.0	2.9	4.9	3.1	2.4	0.5	0.2	93.7	112.3	91.1	65.1	81.4
57 ♂	81	56	250	194	896	4.6	5.2	9.9	8.1	2.1	4.0	3.2	3.2	0.5	0.2	88.1	111.6	84.2	71.5	73.5

Lot no. Adjuvant rat no. and sex	No. days on test	Live wts. and feed consumption: Initial wt. **	Final wt.	Gain in wt.	Total feed eaten	Feed req. per gm. gain in wt.	Blood and tissue wt. relationships: Total weights: Body fat	Liver	Blood	% of body wt.: Body fat	Liver	Blood	Analysis of blood and tissues: Liver fatty acids	Blood fatty acids	Liver non saps	I_2 no. body fat	I_2 no. liver fatty acids	I_2 no. blood fatty acids	I_2 no. liver non saps	I_2 no. blood non saps
		Gms.	Gms.	Gms.	Gms.	Gms.	Gms.	Gms.	Gms.	%	%	%	%	%	%					
S 13																				
Ca. carbonate																				
37 ♀	105	51	167	116	817	7.0	6.6	7.3	3.6	4.0	4.4	2.2	2.9	0.5	0.4	93.9	89.4	106.6	.....	.....
38 ♀	64	55	.....	13	.....	.....	.....	2.5	(dried)	.....	.....	.....	1.7	.....	0.3	.....	99.9	.....	.....	.....
39 ♀	95	47	177	130	828	6.3	4.3	7.5	5.5	2.4	4.2	3.1	3.1	0.5	0.3	106.2	110.7	45.9*	.....	.....
64 ♂	100	44	201	157	936	6.0	2.0	9.7	7.1	1.0	4.8	3.5	3.2	0.3	0.3	98.0	104.7	111.8	.....	.....
63 ♂	100	48	178	130	857	6.6	3.5	9.1	5.0	2.0	5.1	2.8	2.9	0.4	0.4	101.5	129.5	107.1	.....	.....
62 ♂	103	55	241	186	989	5.3	9.1	11.6	7.3	3.8	4.8	3.0	2.7	0.4	0.4	111.6	158.3	94.8	.....	.....
S 14																				
Calcium chloride																				
40 ♀	96	53	174	121	811	6.7	8.4	6.7	5.5	4.8	3.8	3.2	3.1	0.5	0.3	90.4	107.7	58.3	80.5	56.9
41 ♀	104	49	190	141	780	5.5	11.3	9.9	5.0	6.0	5.2	2.6	2.7	0.5	0.3	99.4	169.0	39.3	80.5	81.1
42 ♂	90	50	196	146	852	5.9	3.7	9.5	5.3	1.9	4.8	2.7	2.8	0.4	0.3	100.1	127.6	69.6	84.9	92.8
61 ♀	100	53	204	151	877	5.8	9.9	7.9	5.8	4.9	3.9	2.8	2.2	0.4	0.3	99.3	148.0	33.1	64.6	67.6
68 ♂	98	55	243	188	1048	5.6	8.7	9.9	7.8	3.6	4.1	3.2	3.2	0.5	0.3	100.8	116.0	93.2	79.2	69.1
67 ♂	100	54	238	184	941	5.1	9.3	9.8	7.1	3.9	4.1	3.0	3.2	0.4	0.2	97.8	123.6	97.5	73.5	74.8
41a ♀	86	54	207	153	826	5.4	9.0	12.0	6.5	4.4	5.8	3.1	1.8	0.3	0.3	96.6	160.2	56.1	77.3	51.6
S 15																				
Calcium lactate																				
43 ♀	100	49	168	119	741	6.2	9.1	7.6	4.1	5.4	4.5	2.4	3.0	0.4	0.4	95.3	91.2	107.4	.....	.....
44 ♀	100	53	181	128	818	6.4	9.3	8.6	5.0	5.1	4.7	2.8	3.1	0.3	0.3	109.7	114.0	62.8	.....	.....
45 ♂	103	46	204	158	1009	6.4	3.1	9.2	3.7	1.5	4.5	1.8	3.3	0.6	0.3	97.1	154.1	97.3	.....	.....
66 ♀	98	53	183	130	782	6.0	7.1	7.0	5.0	3.9	3.8	2.7	2.7	0.3	0.6	96.3	139.3	59.6	.....	.....
65 ♂	98	53	186	133	768	5.7	2.9	10.3	5.4	1.6	5.5	2.9	2.7	0.3	0.2	101.2	116.0	63.9	.....	.....
72 ♂	98	57	162	105	804	7.7	2.6	7.6	5.6	1.6	4.7	3.5	3.2	0.4	0.6	100.5	111.6	57.1	.....	.....
S 16																				
Lactose																				
46 ♀	101	57	211	154	1030	6.7	2.8	6.8	6.2	1.3	3.2	2.9	2.8	0.4	0.4	102.4	140.4	102.6	.....	.....
47 ♀	95	57	165	108	924	8.5	0.6*	9.9	5.5	0.4	6.0	3.3	2.8	0.5	0.4	*	137.4	58.7	.....	.....
48a ♂	84	52	163	111	684	6.1	0.9*	8.8	6.1	0.6	5.4	3.7	3.3	0.4	0.4	*	112.6	108.2	.....	.....
71 ♂	95	58	161	103	850	8.2	0.4*	7.0	4.6	0.3	4.3	2.9	2.7	0.4	0.4	*	138.0	100.5	.....	.....
70 ♂	75	53	251	198	910	4.6	4.5	10.5	7.8	1.8	4.2	3.1	3.2	0.4	0.2	97.5	128.2	43.1	.....	.....
69 ♀	87	61	167	106	920	8.7	8.0	8.1	5.4	4.8	4.8	3.2	3.0	0.6	0.4	103.6	115.4	92.9	.....	.....

*All fats under carbon dioxide in refrigerator, which "failed" in hot weather. Gas expanded and popped out stoppers. These fats of very small volume were oxidized before damage was discovered.

**Weights represent those taken after rats had been denied feed for 48 hours.

TABLE 3. DATA PERTAINING TO LIVE WEIGHTS AND FEED CONSUMPTION, BLOOD AND TISSUE RELATIONSHIPS, AND CERTAIN BLOOD AND TISSUE ANALYSES OF RATS FED VARIOUS ADJUVANTS. AVERAGE FIGURES.

Experiment No. 1

Lot no.	No. days on test	Live wts. and feed consumption: Initial wt.	Final wt.	Gain in wt.	Total feed eaten	Feed req. per gm. gain in wt.	Total weights: Body fat	Liver	Blood	% of body weight: Body fat	Liver	Blood	Analysis of blood and tissues: Liver fatty acids	Blood fatty acids	Liver non saps	I_2 no. body fat	I_2 no. liver fatty acids	I_2 no. blood fatty acids
	Av.	Av.	Av.	Av.	Av.	Av.	Av.	Av.	Av.	Av.	Av.	Av.	Av.	Av.	Av.	Av.	Av.	Av.
		Gms.	Gms.	Gms.	Gms.	Gms.	Gms.	Gms.	Gms.	%	%	%	%	%	%			
S 1	82	51	193	135	944	6.7	7.4	8.9	5.8	3.9	4.6	2.9	3.2	0.6	0.1	65.0	103.3	50.7
S 2	86	53	219	167	803	5.2	10.0	10.1	6.5	4.6	4.6	3.0	2.5	0.5	0.3	107.5	136.2	65.0
S 3	95	54	172	118	870	7.6	3.6	8.3	5.3	2.0	4.9	3.1	3.4	0.4	0.3	101.2	91.0*	36.2*
S 4	94	51	216	162	875	5.5	8.4	10.6	6.1	3.8	4.9	2.7	3.3	0.5	0.4	105.7	71.1*	22.5*
S 5	95	50	199	150	865	5.8	7.4	9.4	6.3	3.8	4.7	3.2	3.2	0.4	0.2	110.5	124.6	68.6
S 6	102	54	214	160	951	6.0	8.7	9.3	5.9	4.0	4.3	2.7	3.1	0.5	0.3	106.6	116.7	86.5
S 7	102	54	202	150	964	6.2	5.2	9.0	6.3	2.6	4.4	3.1	3.1	0.4	0.4	97.9	142.1	80.0
S 8	94	54	210	157	924	6.0	5.8	9.8	5.9	2.7	4.6	2.8	3.1	0.5	0.3	96.6	130.7	69.8
S 9	89	54	218	167	770	4.8	12.5	9.5	6.5	5.7	4.4	3.0	4.7	0.7	0.3	108.0	139.8	64.7
S 10	97	52	203	151	963	6.4	5.2	10.0	5.9	2.6	4.9	2.9	3.2	0.4	0.3	96.2	130.0	69.2
S 11	75	51	254	203	825	4.3	11.6	11.7	7.5	4.7	4.5	2.9	2.7	0.4	0.3	99.6	148.4	72.8
S 12	82	54	208	156	767	5.2	5.9	9.6	6.1	2.9	4.6	2.9	3.2	0.5	0.2	94.6	112.4	65.8
S 13	101	50	193	144	885	6.3	5.1	9.0	5.7	2.6	4.7	2.9	3.0	0.4	0.3	102.2	118.5	105.1
S 14	96	53	207	155	876	5.4	8.6	9.4	6.1	4.2	4.5	2.9	2.7	0.4	0.3	97.8	136.0	63.9
S 15	100	52	181	129	820	6.4	5.7	8.4	4.8	3.2	4.6	2.7	3.0	0.4	0.4	100.0	121.0	74.7
S 16	90	56	186	130	886	7.1	2.9	8.5	5.9	1.5	4.6	3.2	3.0	0.4	0.4	101.1	128.7	84.3

*Ether used in extracting fatty acids contaminated.

There was apparently no relationship between the iodine number of the body fats and the sex or the gain in weight of the rats. Neither were the iodine numbers affected by the number of days which the rats were on test. Feeding periods varied in length from 75 to 102 days.

The iodine numbers of the fatty acids of the livers and bloods of the rats apparently bore little relationship to each other or to the iodine numbers of the body fats of the rats, with the exception that in the instances of Lot S 1 (low fat) and Lot S 12 (carotene), the lower iodine values of the body fats seemed to be accompanied by lower iodine values of the fatty acids of the livers of the rats.

After the iodine values of the nonsaponifiable fractions of the livers and bloods of a number of the lots of rats had been determined it appeared that further determinations of this sort would add little of value to this problem and therefore they were discontinued.

EXPERIMENT II

From an analysis of the foregoing results it appeared that further study of the feeding of carotene might be of value, and a second experiment therefore was set up in part for this purpose. In 1938, S. M. Das Gupta (8) reported that "the feeding of carotene to cows seemed to result in an *increase* in the Reichert and iodine numbers of their butter fat, indicating that carotene may play some part in the oxidation and *desaturation* of fatty acids in the animal body." Since a great deal of research (12a) had shown that choline and niacin greatly influenced the deposition of fat in the livers of rats, it was decided to feed two lots of rats in this experiment choline chloride and niacin as adjuvants to their diet.

PROCEDURES

In this experiment six lots of seven rats each, all males, were individually caged, fed and attended, as described in Experiment I. The basal diet fed differed from that used in Experiment I in that corn starch replaced sucrose and 1 percent of yeast vitamin concentrate (Harris) was fed instead of 2 percent of yeast. The composition of diets fed is shown in table 1.

The carotene feeding solution was prepared by mixing 1 gram of carotene in 432 drops of soybean oil. Some of the carotene went into solution while the remainder stayed in suspension. This carotene suspension was thoroughly stirred and shaken each time it was fed. Three times weekly each rat in Lot S 20 received 1 drop of the carotene suspension, the rats in Lot S 21 each received 2 drops and the rats in Lot S 22 each received 4 drops. Thus, since the same dropping pipette was used in all operations, the rats in Lot S 22 received approximately twice as much

carotene and the rats in Lot S 20 one-half as much carotene as did the rats in Lot S 21, and approximately the amounts of carotene in their diets as specified in table 1. The rats in Lot S 21 received about the same amount of carotene weekly as was fed the rats in Lot S 12 in Experiment I (see table 1).

The rats were kept on test from 73 to 79 days. At the end of this time they were anesthetized with ether and the intestinal tracts removed. The bloods and livers of these rats were not removed, but instead the entire body of each rat minus the intestinal tract was frozen and then ground up and rendered in the autoclave.

The samples of extracted body fat were not, as in the first experiment, stored under carbon dioxide until analyzed, but instead were stored in dry ether solution. This small volume of ether (50 ml.) was removed at room temperature in a vacuum desiccator at the time of analysis. Thus, the possibility of the oxidation of the fat in the event of the failure of the refrigerator, such as occurred in the case of a few samples of the preceding experiment, was minimized.

RESULTS

The rats used in this experiment grew much more uniformly than those used in Experiment I and there were few outstanding differences among them in rate of growth or appearance.

The data obtained in this experiment are compiled in tables 4 and 5. An examination of table 4 shows that there was less divergence between the iodine numbers of the rats within a lot than there was in the first experiment.

The body fats which exhibited the lowest average iodine values were those of the rats in Lot S 20 (.0070 gm. carotene), whereas the body fats of the rats of Lots S 21 (.014 gm. carotene) and S 22 (.028 gm. carotene) presented somewhat higher average iodine values, approximating those of the body fats from the rats in Lot S 17 (basal diet). There was no evidence that the feeding of choline chloride or niacin had influenced consistently the hardness of the deposited body fat.

DISCUSSION

No single explanation can be offered for the divergence of the results obtained in Experiments I and II from the feeding of carotene. In the first experiment sucrose was fed, whereas in the second experiment starch had replaced the sucrose. Workers in the field of vitamins, especially those who have investigated the B complex vitamins, have found that rats which received starch in their diets did not produce the same results as those which received sucrose. Differences of function between starch and sucrose in the diet of rats were also apparently found by Griffith (12A) in his studies of the choline requirements of rats.

TABLE 4. DATA PERTAINING TO LIVE WEIGHT AND FEED CONSUMPTION; AND THE IODINE NUMBERS OF THE BODY FAT OF RATS FED VARIOUS ADJUVANTS. EXPERIMENT NO. II.

Lot no. Rat no. Adjuvant	Days on test	Initial body wt.**	Final body wt.	Gain in body wt.	Total feed eaten	Feed eaten per gm. gain	I_2 no. body fat
		Gms.	Gms.	Gms.	Gms.	Gms.	
S 17 Basal diet							
1	77	64	266	202	984	4.9	107.6
2	77	62	289	227	1022	4.5	107.2
3	77	64	295	231	988	4.3	104.3
4	77	73	330	257	1116	4.3	106.1
5	77	71	295	224	1057	4.7	107.8
6	77	67	280	213	991	4.6	107.9
7	77	66	285	219	987	4.5	103.4
S 18 Choline							
8	78	65	301	236	1055	4.5	103.4
9	78	63	249	186	895	4.8	105.2
10	78	63	324	261	990	3.8	105.2
11	78	70	305	235	975	4.1	101.1
12	78	60	273	213	962	4.5	100.5
13	78	64	266	202	897	4.4	99.7
14	78	66	318	252	1036	4.1	101.1
S 19 Niacin							
15	79	65	250	185	931	5.0	101.3
16	76	79	363	274	1130	4.1	103.5
17	76	70	247	177	832	4.7	102.4
18	76	66	293	227	978	4.3	104.0
19	70	67	287	220	863	3.9	103.4
20	76	64	318	254	1078	4.2	103.6
21	73	67	279	212	917	4.3	102.5
S 20 Carotene							
22	77	66	297	231	983	4.2	99.3
23	77	72	306	234	1097	4.7	99.6
24	77	78	296	218	1011	4.6	102.7
25	73	63	231	168	747	4.4	97.1
26	77	65	324	259	1086	4.2	101.1
27	77	71	303	232	1023	4.4	104.9
28	77	70	313	243	1051	4.3	101.6
S 21 Carotene							
29	78	69	310	241	1028	4.3	107.8
30	78	80	341	260	1075	4.1	109.5
31	*	*	*	*	*	*	*
32	78	71	304	233	959	4.1	108.6
33	78	69	305	236	985	4.2	107.2
34	78	74	255	181	815	4.5	107.3
35	78	71	260	189	866	4.6	107.0
S 22 Carotene							
36	79	80	286	206	913	4.4	107.3
37	79	76	335	259	1106	4.3	108.8
38	75	72	308	236	1000	4.2	108.2
39	79	78	279	201	890	4.4	107.5
40	79	75	309	234	1031	4.4	105.0
41	79	78	266	188	910	4.8	104.4
42	79	77	300	223	984	4.4	103.9

*Rat No. 31 became vicious and unmanageable and was eliminated.
**Weights represent those taken after rats had been denied feed for 48 hours.

TABLE 5. DATA PERTAINING TO LIVE WEIGHT AND FEED CONSUMPTION; AND THE IODINE NUMBERS OF THE BODY FAT OF RATS FED VARIOUS ADJUVANTS. AVERAGE FIGURES. EXPERIMENT NO. II.

Lot no. Adjuvant	Days on test	Initial body wt.** av.	Final body wt. av.	Gain in body wt. av.	Total feed eaten av.	Feed eaten per gm. gain av.	I_2 no. body fat av.
		Gms.	Gms.	Gms.	Gms.		
S 17 basal diet	77	67	291	225	1021	4.5	106.3
S 18 basal diet + 1% choline chloride	78	64	291	226	973	4.3	102.3
S 19 basal diet + 1% niacin	75	68	291	221	961	4.4	103.0
S 20 basal diet + 0.0070 gm. carotene weekly	76	69	296	226	1000	4.4	100.9
S 21 basal diet + 0.0140 gm. carotene weekly	78	72	296	223	955	4.3	107.9
S 22 basal diet + 0.028 gm. carotene weekly	79	77	298	221	976	4.4	106.5

**Weights represent those taken after rats had been denied feed for 48 hours.

In the first experiment, the carotene was fed in a vehicle of coconut oil. While it was appreciated that the inclusion of any considerable amount of coconut oil in the diet of rats would lower the iodine values of their body fats, it was considered in the light of Schoenheimer's work on the rapid turnover of fats in the body, that the small amount of coconut oil fed would not influence significantly the results obtained. Each rat received only 0.50 gram of coconut oil per week, as against 6 to 9 grams of soybean oil. If each rat, contrary to the work of Schoenheimer, Sinclair and others, had selectively stored all the coconut oil fed during the progress of the experiment it would have amounted to less than 6 grams per rat, as against 68 to 90 grams of soybean oil which these rats had ingested. If the small inclusions of coconut oil could have influenced the body fat deposited a further study of this effect might be worthwhile.

The work of Basu (4A) which showed that the absorption of carotene from the intestinal tract was dependent upon the simultaneous presence of a suitable fat or oil also might possibly be considered in this connection. In both of the experiments here reported, however, the concentration of soybean oil in the intestinal tracts of the rats which received the carotene should have made the conditions there, as far as the oil content was a factor, approximately the same in both experiments, even though in the first experiment the carotene was dissolved in a modicum of coconut oil. Of interest, too, are the papers by Monaghan and Schmitt (18), by Sumner (25A), by Quackenbush, Cox and Steenbock (19) and by Hove (16A), which are concerned with the oxidation of carotene in vitro under certain special conditions. It is not, however, probable that any of the considerations specified in these papers would apply to the experiments as conducted in this research.

It would seem that ordinarily this relationship of carotene to fat metabolism might be worthy of immediate further study. Also, since in the first trial some of the other adjuvants appeared to affect the firmness of the body fats deposited further experimentation with these might also provide some data contributing to a fuller understanding of the fundamentals of fat metabolism. However, if the data obtained with rats are indicative of what might be expected in practice if pigs were fed rations containing these adjuvants, it does not appear that this study would lead to results of practical value in the solution of the problem of "soft" pork.

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THE FECAL EXCRETION OF LIPIDS BY RATS AS INFLUENCED BY THE DIET[1]

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(Received for publication November 21, 1949)

There has not, apparently, been a great deal of research conducted on the effect of various adjuvants to the diet of rats upon the digestibility and excretion of fats. Boyd, Crim and Lyman ('32), who reported upon the digestion and absorption of calcium soaps by rats, presented a good bibliography of earlier work concerned with the effect of calcium salts upon fat absorption. Steenbock and associates ('35) found that bile, sodium benzoate, alcohol, peptone and cane sugar decreased fat absorption in rats; and Frazer ('46) reported the effect of choline on the intestinal absorption of fat. Barnes, Primrose and Burr ('44) found that while the absorption of a fat such as steam lard was influenced only slightly, lard that contained hydrogenated fat was digested more completely when the protein of the diet approximated 30% than when only a 14% protein diet was fed.

Deuel and associates (Deuel and Hallman, '40a; Deuel et al., '40b; Augur et al., '47; Crockett and Deuel, '47), Hoagland and Snider ('40, '43), Mattil ('46), Burr and Barnes ('43b), and Wikoff, Koonce and McGuire ('43) have reported extensively on the digestion by rats of artificial, natural and hydrogenated fats, whereas Deuel and Hallman ('40a) and Mattil and Higgins ('45) have studied the digestibility of certain artificial triglycerides.

[1] Journal paper no. J1721 of the Iowa Agricultural Experiment Station. Project no. 507.

Levin and Smith ('27) have reported that rats were able to utilize 98 and 99% of the fat in their diets, as measured by the ether extract of their feces, when the diets contained from 22 to 64% lard, and the work of Bloor ('38, '39), Burr and Barnes ('43a) and Eckstein ('41) has apparently established the fact that the normal fecal lipid excretion of rats, like that of most other species, while influenced by the amount and kind of fat in the diet, is not entirely dependent upon it.

Numerous researchers have studied the effect of lactose in the diets of rats, but these studies have been primarily concerned with the relative nutritional value of the sugars, the production of cataracts, the effect of a change in the hydrogen ion concentration of the intestinal tract on the absorption of calcium and phosphorus, or the effects of lactose on gastrointestinal motility, without specific reference to the lipids. These latter studies will be considered later. There have also been several studies on the effect of crude fiber in the diet upon gastrointestinal motility and the excretion of certain nutrients, but a specific study of lipid excretion does not seem to have to been an objective.

A few years ago the present authors reported the effects of various adjuvants to the diet of rats on the changes in body fats induced by the feeding of soybean oil (Schulz and Thomas, '45). The feces obtained from the rats during these experiments were collected daily and stored in metal containers. The present article is a report of the effect these various diet adjuvants had upon the excretion by the rats of fatty acids and non-saponifiable lipids.

EXPERIMENTAL

The study consisted of two experiments. In the first experiment 13 lots of 6 weanling rats each (three males and three females), averaging 56 gm in weight, each rat individually caged and attended, were fed a diet composed of 50% solvent-processed soybean oil meal, 1.5% salt mixture, 2% yeast or 1% yeast concentrate,[2] 10% soybean oil, cod

[2] Harris; from the Harris Laboratories, Tuckahoe, N. Y.

liver oil concentrate,[3] and cane sugar to make 100%. Lot 2 received this basal ration; lot 1 received no soybean oil; lot 9 received 30% soybean oil; lot 16 received lactose instead of sucrose; and to the basal diet of the other lots certain adjuvants which might have some effect upon fat metabolism were added. The different adjuvants replaced equal weights of sugar where more than 1% of the adjuvant was fed; otherwise the adjuvant was merely added to the basal ration.

In experiment 1 the rats were kept on test until they reached a weight of 250 gm or had ceased growing. Because of the nature of the diets fed, the rats both within and among the different lots grew at rather widely different rates; so that when the experiment was terminated, the number of days the different rats were on test varied considerably. The rats used in experiment 1 were on test an average of 95 ± 4.0 days. Their final weight averaged 205 gm and their average weight gain was 152 gm each. It was necessary for various reasons to discard one rat from each of lots S9, S13 and S16. Therefore there were only 5 rats in each of these lots at the close of the experiment.

In the second experiment 6 lots of 7 weanling rats each, all males averaging 70 gm in weight and managed as indicated above, were fed the same basal diet as those in experiment 1 except that corn starch replaced the cane sugar. These animals grew much more uniformly than did the rats used in experiment 1; they had attained an average body weight of 294 gm, with an average weight gain of 224 gm each, when they were taken off experiment after an average of 77.8 ± 0.3 days. Lot 23 contained only 6 rats at the close of the experiment.

Further details concerning the nature of the experiments and the management of the rats are recorded in Research Bulletin 336 of the Iowa Agricultural Experiment Station (Schulz and Thomas, '45). Table 1 lists the diets fed in these experiments.

[3] Upjohn.

TABLE 1

Adjuvants fed and amount of each which was added to or which replaced sugar or starch in the basal diets

LOT NO.	ADJUVANT AND AMOUNT IN DIET		LOT NO.	ADJUVANT AND AMOUNT IN DIET	
		Experiment 1			
S1	Positive control	No soybean oil	S9	Negative control	30% soybean oil
S2	Basal diet	....	S10	Agar-agar	10%
S3	Charred alfalfa meal	20%	S11	Casein	20%
S4	L-Cystine	0.5%	S12	Carotene (per rat)	0.0140 gm per wk.
S5	DL-Methionine	0.5%	S13	Calcium carbonate	1st mo. 2%, then 1%
S6	Ascorbic acid (per rat)	25 mg per day	S14	Calcium chloride	1st mo. 2.2%, then 1.1%
S7	Bile salts	0.5%	S15	Calcium lactate	1st mo. 6.2% then 3.1%
S8	Sodium fluoride	0.05%	S16	Lactose replaced all sucrose	36.5%
		Experiment 2			*gm per rat per wk.*
S19	Basal diet	....	S22	Carotene	0.0070
S20	Choline chloride (Merck)	1%	S23	Carotene	0.0140
S21	Niacin	1%	S24	Carotene	0.0280

BASAL DIETS [1]	EXPT. NO. I	EXPT. NO. II
Soybean oilmeal (solvent-extracted = 0.5% fat)	50	50
Soybean oil	10.0	10.0
Salt mixture ($CaCO_3$ 55; bone meal 20; NaCl 20; ferric citrate 4.5; KI 0.05% [2])	1.5	1.5
Yeast	2.0	0.0
Yeast concentrate	0.0	1.0
Cane sugar	36.5	0.0
Starch	00.0	3.75
Total	100.0%	100.0%

[1] Each rat received weekly three drops of cod liver oil concentrate.
[2] Lot 14: salt mixture = $CaCl_2$ 55; $Ca_3(PO_4)_2$ 20; NaCl 20; ferric citrate 4.5; KI 0.05%.

All the feces obtained from each rat were collected each day throughout the duration of the experiment, and these composited feces were stored in light-proof tin containers. Previous to analysis each can of feces was ground to pass

TABLE 2

Average feed ingested and feces excreted daily

LOT NO. AND ADJUVANT FED		AVERAGE FEED INGESTED DAILY	AVERAGE LIPIDS INGESTED DAILY	AVERAGE FECES EXCRETED DAILY	% FECES EXCRETED [1]
		gm	*gm*	*gm*	
		Experiment 1			
S1	Low fat-sucrose diet	10.78	0.05	1.20	11.1
S2	Basal sucrose diet	9.52	1.00	1.20	12.7
S3	Charred alfalfa	9.16	1.00	2.71	29.5
S4	L-cystine	9.36	0.98	1.15	12.3
S5	DL-methionine	9.17	0.96	1.08	11.8
S6	Ascorbic acid	9.37	0.98	1.09	11.7
S7	Bile salts	9.47	0.99	1.26	13.4
S8	Sodium fluoride	9.94	1.04	1.20	12.1
S9	30% soybean oil	8.57	2.61	0.97	11.3
S10	Agar-agar	9.90	1.04	2.06	20.8
S11	Casein	11.28	1.19	1.35	12.0
S12	Carotene	9.59	1.08	1.19	12.5
S13	$CaCO_3$	8.81	0.92	1.23	13.9
S14	$CaCl_2$	8.87	0.93	1.07	12.0
S15	Calcium lactate	8.23	0.86	1.03	12.5
S16	Lactose	10.32	1.08	1.74	16.7
Standard error of a mean (6 rats)		± 0.50	± 0.059	± 0.09	± 0.49
Standard error of a mean (5 rats)		± 0.55	± 0.065	± 0.10	± 0.54
		Experiment 2			
S19	Basal starch diet	13.26	1.39	0.79	6.0
S20	Choline	12.47	1.31	0.78	6.3
S21	Niacin	12.79	1.34	0.83	6.5
S22	Carotene	13.06	1.37	0.91	7.0
S23	Carotene	12.24	1.30	0.89	7.3
S24	Carotene	12.36	1.32	0.88	7.1
Standard error of a mean (7 rats)		± 0.41	± 0.043	± 0.03	± 0.13
Standard error of a mean (6 rats)		± 0.44	± 0.046	± 0.03	± 0.14

[1] Weight feces × 100/weight of feed ingested.

a 60-mesh sieve and thoroughly mixed. The fatty acids and non-saponifiable lipids were then determined upon separate 2-gm aliquots of the feces as follows: 2 gm of ground feces, 4 gm of solid KOH, 5 ml water and 35 ml C_2H_5OH (95%) were placed into 250-ml erlenmeyer flasks and these flasks, fitted with cold finger condensers, were placed upon a steam hot plate. After the saponification had continued for 4 hours, the condensers were removed, water was added and the liquids in the flasks were evaporated to small volumes to remove most of the alcohol. The residues were then washed with water into 250-ml separatory funnels and extracted repeatedly with diethyl ether to remove the non-saponifiable lipids. The combined ether extracts were permitted to evaporate to dryness at laboratory temperature; the residues were dissolved in diethyl ether and filtered into 50-ml erlenmeyer flasks which contained anhydrous sodium sulfate. These flasks were then stoppered and placed in a refrigerator. After several hours, the dried ether extracts were filtered from the sodium sulfate, allowed to evaporate to dryness, again taken up in anhydrous diethyl ether and filtered into tared beakers. The residues after evaporation at room temperature were dried to constant weight at 70°C. in a vacuum oven and reported as non-saponifiable lipids.

The fatty acids were determined in the same manner except that the alkaline saponifications of the rat feces were acidfied with 20% HCl before being washed into separatory funnels and extracted with ether. Since the residues obtained in this case consisted of both the fatty acids and the non-saponifiable lipids, the weight of the non-saponifiable lipids, obtained first, was subtracted to obtain the weight of the fatty acids. All determinations were made in duplicate and where results were not satisfactory further replications were made.

RESULTS

Table 2 lists the average amounts of feed ingested and feces excreted daily by the different lots of rats in both experiments 1 and 2. In experiment 1, the basal diet that

contained 10% soybean oil (lot 2) is considered the diet that provided "normal" feces, whereas in experiment 2, in which starch replaced the sucrose in the diet, the rats in lot S19 received the basal starch diet.

If the "weight of the feces excreted" × 100/"weight of feed ingested" is taken as the percentage of feces excreted, it is found that in experiment 1 the addition of 20% of charred alfalfa or the addition of 10% of agar-agar to the diet, or the replacement of the sucrose in the diet by lactose, resulted in an increased percentage excretion of feces. Statistical analyses of the figures[4] show that since the rats in lot S2 excreted 12.3% feces, 14.7% excretion is the necessary mean to be judged significantly greater than this. The rats in lots S1, S5, S6, and S9 excreted smaller percentages of feces than did the rats in lot 2, but these differences were not significant. Apparently the inclusion of L-cystine, DL-methionine, ascorbic acid, bile salts, sodium fluoride, casein, calcium carbonate, calcium chloride or calcium lactate in the basal diet of the rats used in experiment 1, in the amounts here specified, had no statistically significant influence on the percentage of fecal excretion by these rats. This percentage was also not influenced by the exclusion of the 10% soybean oil in the basal diet nor by the increase of the soybean oil to 30% of the diet.

In experiment 1 the rats which received carotene in their diet (lot S12) did not excrete a percentage of feces significantly different from that of those rats which received the basal sucrose diet (lot S2). In experiment 2 statistical analysis shows that at the $P = 0.01$ level all the lots of rats that received carotene at different levels of the diet (lots S22, S23 and S24) excreted increased percentages of feces when compared with the lot of rats which received the basal starch diet (lot S19).

Since experiments 1 and 2 were conducted at different times, it is not statistically valid to compare them. However,

[4] Grateful acknowledgment is made to Mr. J. G. Darroch of the Statistical Laboratory of the Iowa State College for statistical analyses.

it seems worthy of note that the rats used in experiment 1 excreted approximately twice the percentage of feces excreted by the rats used in experiment 2.

Table 3 lists the figures showing the extent of the excretion and the apparent retention of dietary lipids by the

TABLE 3

Excretion and retention of lipids in the diet

LOT NO.	AVERAGE WT. F.A. IN FECES DAILY [1]	AVERAGE WT. N.S.L. IN FECES DAILY [1]	AVERAGE WT. TOTAL LIPIDS APPARENTLY RETAINED DAILY	PER CENT LIPIDS APPARENTLY RETAINED DAILY
	gm	*gm*	*gm*	%
		Experiment 1		
S1	0.047	0.031	(—) 0.026	
S2	0.072	0.035	0.892	89.3
S3	0.112	0.044	0.838	84.4
S4	0.070	0.032	0.881	89.5
S5	0.068	0.034	0.860	89.5
S6	0.073	0.033	0.873	89.3
S7	0.075	0.034	0.884	89.0
S8	0.072	0.033	0.937	90.0
S9	0.062	0.038	2.514	96.2
S10	0.062	0.038	0.942	90.4
S11	0.077	0.036	1.082	90.6
S12	0.079	0.035	0.963	89.5
S13	0.063	0.030	0.831	90.0
S14	0.070	0.028	0.833	89.5
S15	0.056	0.022	0.787	91.0
S16	0.098	0.031	0.952	88.1
S.E.[2]	(6) ± 0.0075	± 0.0028	± 0.0538	± 0.59
S.E.[2]	(5) ± 0.0082	± 0.0030	± 0.0590	± 0.65
		Experiment 2		
S19	0.046	0.021	1.320	95.1
S20	0.047	0.020	1.238	94.8
S21	0.051	0.021	1.267	94.6
S22	0.059	0.020	1.295	94.2
S23	0.060	0.021	1.214	93.7
S24	0.059	0.019	1.245	94.1
S.E.[2]	(7) ± 0.0031	± 0.0014	± 0.0412	± 0.19
S.E.[2]	(6) ± 0.0033	± 0.0016	± 0.0446	± 0.20

[1] F.A. = fatty acids; N.S.L. = non-saponifiable lipids.

[2] S.E. (5), (6) or (7) = standard error of a mean obtained by using results from 5, 6 or 7 rats.

rats in both experiments 1 and 2. Statistical analysis using $P = 0.01$ shows that in experiment 1 the inclusion of 20% charred alfalfa in the diet (lot 3) significantly decreased the apparent percentage retention of the lipids, and that the substitution of 30% soybean oil in place of the 10% soybean oil contained in the basal ration significantly increased the apparent percentage retention of lipids when the apparent percentage retention is compared with that established by the rats which received the basal ration (lot 2). The rats in lot 1, having received no added oil in their low fat diet, excreted more lipids than they ingested and thus established an apparent negative percentage retention. The inclusion of 10% agar-agar (lot 10) and the substitution of lactose for sucrose in the diet (lot 16), like the inclusion of charred alfalfa, resulted in an increased excretion of feces but had no significant effect upon the percentage of lipids apparently retained by the rats which received these diets.

However, if the figures are calculated for the ratio of fecal excretion of fatty acids to fecal excretion of non-saponifiable lipids (fecal F.A./fecal N.S.L.), it is found that this ratio for the feces produced by the rats that received the basal diet (lot S2) is 2.13 ± 0.25, whereas for the feces produced by the rats that received the lactose diet (lot 16) the ratio is 3.24 ± 0.42. These ratios differ significantly; calculations also show that the inclusion of charred alfalfa in the diet apparently increased this F.A./N.S.L. ratio slightly, while the exclusion of soybean oil, the inclusion of 30% soybean oil and the inclusion of 10% agar-agar, respectively, in the basal diets apparently decreased this F.A./N.S.L. ratio when compared to the ratio established by the rats in lot 2. These latter differences were not, however, large enough to be significant and are cited merely as possible factors which might under certain conditions affect the differential excretion of the fatty acids and the non-saponifiable lipids.

Statistical analysis using $P = 0.01$ shows that in experiment 2 the rats which received the three levels of carotene

in their diets (lots 22, 23, and 24) apparently retained significantly decreased percentages of total lipids when compared with the rats in lot 19, which received the basal starch diet.

DISCUSSION

On the basis of these experiments it appears that the fecal excretion of the rats was increased by the inclusion of charred alfalfa and agar-agar and by the substitution of lactose for sucrose in a sucrose diet. Of the three, however, only the inclusion of charred alfalfa resulted in an alteration of the apparent percentage retention of lipids. The charred alfalfa was found by analysis to contain 29.7% crude fiber, whereas the same analytical method showed only 0.80% crude fiber in the agar-agar.

Many investigators have studied the effects of varying percentages of lactose in the diet of rats upon gastric motility. While the results in some cases were controversial, there seemed to be general agreement that diarrhea of varying degrees of intensity resulted when lactose constituted more than 20% of the diet. A few of these investigators considered the effects of lactose upon digestibility of the organic nutrients in the diet, and concluded that the feeding of lactose resulted in impairment of the digestion of certain organic nutrients and in an increased excretion of food residues. The subject of the effect of lactose upon the gastrointestinal motility of various species has recently been very capably reviewed by Fischer and Sutton ('49).

The rats in lot 16 of the present experiments, which received the lactose diet, all developed a diarrhea of varying degrees of intensity, but this diarrheal condition moderated and then ceased within a period of a week, after which the feces were hard, black and brittle when dry. We agree with those investigators who found that lactose in the diet increased the percentage of feces excreted, but are not prepared to state whether or not this condition is the result of food residues. As is stated above, although the lactose diet increased the ratio of fatty acids to non-saponifiable

lipids excreted, it did not significantly affect the apparent percentage retention of total lipids.

As was noted previously, while it is not statistically valid to compare the results obtained in experiments 1 and 2, it seems desirable to call attention to the fact that, in addition to the differences in fecal excretion, there were also differences that resulted when carotene was fed in the two experiments. In experiment 1 the addition of carotene to the sucrose diet resulted in a decreased iodine number for the body fat of the rats (Schulz and Thomas, '45) but in no alteration in the amount of feces excreted or lipids retained.

In experiment 2 the inclusion of carotene in the basal starch diet at three levels resulted in no change in the iodine numbers of the body fats (Schulz and Thomas, '45), in an increased percentage of feces excreted, and in an apparently decreased percentage of lipids excreted when compared with the fecal excretion of the rats that received the basal starch diet.

SUMMARY

In two experiments in which rats were fed various adjuvants to basal diets, the feces were quantitatively collected and analyzed for fatty acids and non-saponifiable lipids.

In the first experiment, in which a basal sucrose diet that contained 10% soybean oil was fed, the addition of L-cystine, DL-methionine, ascorbic acid, bile salts, sodium fluoride, carotene, calcium carbonate, chloride or lactate had no significant effect upon the percentage of fecal excretion or the percentage retention of lipids.

The percentage excretion of feces was increased by the inclusion of 20% charred alfalfa or 10% agar-agar and by the substitution of lactose for the sucrose in the diet. The apparent percentage retention of total lipids was decreased by the inclusion of the charred alfalfa and increased by feeding 30% of soybean oil. The elimination of added soybean oil from the diet resulted in an apparent negative retention of lipids.

In the second experiment, in which starch replaced the sucrose of the basal diet, the addition of niacin or choline chloride had no effect on fecal elimination or retention of lipids. However, in this experiment the addition of carotene at three levels resulted in a significantly increased percentage of fecal elimination and in a significantly decreased percentage of total lipids apparently retained.

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Growth Inhibitory Effect of Certain Polysaccharides for Chickens

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(Received for publication March 17, 1964)

IT IS well-known that the growth of chickens is inhibited if their diet contains either linseed meal (Kratzer and Williams, 1948; MacGregor and McGinnis, 1948), or ground carobs (Kratzer and Williams, 1951; Bornstein *et al.*, 1963), or guar meal (Borcher and Ackerson, 1950; Vohra and Kratzer, 1964). In each case, the feces of the chickens were extremely sticky and tended to paste up the vent. Linseed meal loses its growth-inhibitory properties if it is given a preliminary water treatment; or if the pyridoxine content of the diet is increased (Kratzer and Williams, 1948; MacGregor and McGinnis, 1948). Similar treatment of ground carobs

Poultry Science 43(5): 1164-1170, 1964.

... either very slight but significant ... and Williams, 1951), or no growth improvement (Bornstein *et al.*, ...) of chicks. Our unpublished studies indicate that guar meal is not improved either by treatment with water or by the addition of pyridoxine.

Linseed meal, carob and guar seeds are excellent sources of polysaccharides which are classified as gums and mucilages. An obvious question in considering these materials is whether this growth depressing effect is due to the high levels of polysaccharides which they contain.

A part of the growth inhibitory properties of linseed meal has been attributed to its mucilage content (Mani *et al.*, 1949; Clagett *et al.*, 1955) even though the latter workers doubted this in another report (Schlamb *et al.*, 1955). A depression in the growth of rats has been observed when their diets contained more than 5% agar agar or Irish moss (Nilson and Schaller, 1941). The digestibility of agar agar, pectin and guar gum is reported to be 21%, 19% and 76% respectively, and no significant depression in the growth of rats was observed when the diets contained these polysaccharides at levels of 15%, 10% and 6%, respectively (Booth *et al.*, 1963). Ershoff and Wells (1962) found no significant depression in the growth of rats which were fed 10% pectin, guar gum, locust bean gum or carrageenan. Carrageenin appears to interfere with the digestion of proteins by pepsin under certain conditions (Vaughan *et al.*, 1962). The present work which was reported in abstract form (Kratzer and Vohra, 1963) is a study of the effects of feeding polysaccharides on the growth of chickens.

EXPERIMENTAL

Commercially prepared samples of the following polysaccharides were used in this ..., guar gum,[2] locust (or carob) gum,[1] gum arabic,[1] gum ghatti, gum tragacanth,[1] gum karaya, pectin, carrageenin, cellulose,[3] methylcellulose, sodium salt of carboxymethylcellulose, agar agar, dextrin, dextran and polygalacturonic acid.[2]

Dried okra powder was prepared by homogenizing fresh okra in a blender and an excess of methanol was added to the homogenate. The mixture was stirred and filtered on a Büchner funnel under vacuum. The solid residue was dried in a current of air at room temperature, and ground.

To obtain mucilage, linseed oil meal was soaked in an excess of water overnight, stirred and filtered through muslin. The volume was reduced by leaving the mucilage in shallow pans in a draft of air. The polysaccharide was precipitated from the mucilage solution by the addition of methanol. It was filtered and dried in a current of air at room temperature.

Caramel was prepared by the heating of sugar crystals (sucrose) in an iron crucible over a burner.

Psyllium husk and dried kelp were obtained from commercial sources.

The polysaccharides were added to the stock diet (Table 1) at the required level without making any adjustment in its composition (Table 1) at the required level without making any adjustment in its composition. Day-old, Arbor-Acres broiler type chicks were weighed and distributed into groups of approximately equal average weight. They were housed in electrically heated batteries in which water and feed were available *ad libitum*. The chicks were weighed twice every week as groups and individually at the termination of the experiment.

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[1] Samples through courtesy of Stein, Hall and Co., Inc.

[2] Through courtesy of Dr. M. A. Joslyn.

[3] Solka-floc, Brown Co., Berlin, New Hampshire.

TABLE 1.—*Composition of the stock diet*

Ingredient	Inclusion, gm./kg.
Ground milo	175
Ground corn	157
Ground barley	200
Bran, wheat	150
Soybean meal, 44% protein	120
Fish meal	75
Meat and bone meal, 50% protein	12
Whey, dried	25
Milk, skimmed, dried, 35% protein	25
Alfalfa meal	40
Limestone, ground	15
Salt*	5
Vitamins†	1

* Contains 0.025 gm. manganese sulfate (70% feed grade).

† Supplies: riboflavin, 1.1 mg.; niacin, 1.1 mg.; Ca-pantothenate, 1.1 mg.; choline chloride, 5.5 mg.; sulfaquinoxaline, 125 mg.; vitamin A, dry (20,000 I.U./gm.), 375 mg.; vitamin D_3, dry (4,500 I.C.U./gm.), 250 mg.; and folic acid, 33.3 μg.; and bran to make 1 gm.

A slurry of 120 gm. guar gum in 1500 ml. of water was reacted overnight at 37°C. with 100 ml. of a solution containing 6 gm. of either Cellulase-100[1], or the isolated enzyme from the sprouted guar beans (Vohra and Kratzer, 1964). The mucilage broke down in this process and a fluid was obtained which was reduced to about half its volume in shallow pans in a current of air at room temperature. This concentrated solution was mixed with 5880 gm. of stock diet and the diet was fed as such without any further drying.

Pectin (240 gm.) was stirred in 2 liters of water to give a thick, viscous mass which was reacted with 4.8 gm. of pectinase in 100 ml. water at 37°C. for about 12 hours. The viscous mass was stirred frequently and yielded a fluid solution which was concentrated to about 800 ml. in shallow pans in a draft of air and mixed with 5760 gm. stock diet to feed the chicks. It was not dried further.

The relative growth value compares the final average weight of the chickens fed the experimental diet with the average weight of chickens fed the stock (control) diet in the same experiment. For the comparison, the relative weight of the group fed the control diet is adjusted to 100.

[1] Through courtesy of Miles Chemical Co.

TABLE 2.—*Relative growth (R.G.) and gm. feed/gm. gain (F/G) of chickens fed various polysaccharides in their diets*

Experiment Series	1	2	3	4	5	
Duration of trial, days	20	21	20	21	20	
Polysaccharide and its level	R. G.	R. G.	R. G.	R. G.	R. G.	F/G
Control	100	100	100	100	100	1.73
Cellulose, 2%	101	92				
Guar gum, 2%	67.4	64		61		
Guar gum, 1%	84.4	80		72.2	79.1	1.72
Guar gum, 0.5%				78.6	92	[illegible]
Guar gum, 0.25%				89.1	93.4	[illegible]
Locust gum, 2%	73	73.6				
Locust gum, 1%			96.8			
Locust gum, 0.5%			87.8			
Locust gum, 0.25%			96.5			
Gum tragacanth, 2%	65.8	66				
Gum arabic, 2%	96.5	85.6				
Dextran, 2%	94.8					
Dextrin 2%		103				
Pectin, 4%	88.6	77.5		89.5		
Pectin, 2%	92.4	86.5				
Kelp, 4%			99.5			
Kelp, 2%			99.5			
Actual gain of control, gm.	256	330	332	294	297	

TABLE 3.—*Relative growth (R.G.) and mg. powered 100 gm. body weight (P/B.W.), gm. feed/gm. gain (F/G), of chickens fed various polysaccharides in their diets*

Experiment Series	6		7		
Duration of trial, days	20		20		
Polysaccharide, and its level	R. G.	P/B.W.	R. G.	P/B.W.	F/G
Control	100	375	100	342	1.54
Cellulose, 2%	96.8				
Okra, dried, 2%	87.6	470	81.8	462	1.84
Carrageenin, 2%	75	532	75.4	555	1.91
Linseed mucilage, 2%	95.4	370	97.4	—	1.84
Psyllium husk, 2%	87.4	435	81.8	456	1.92
Methylcellulose, 2%	103				
Carboxymethylcellulose, (Na), 2%	96.5	360	95.8	—	1.88
Caramel, 2%	100				
Actual gain, gm.	339		320		
Control			100	474	1.78
Kelp, 2%			114	506	1.59
Agar agar, 2%			110	415	1.72
Gum karaya, 2%			72	432	1.67
Gum ghatti, 2%			104.5	427	1.81
Actual gain, gm.			280		
Control			100	410	1.78
Gum ghatti, 2%			95		1.81
Gum karaya, 2%			70	500	1.92
Agar agar, 2%			102		1.87
Actual gain, gm.			315		

RESULTS AND DISCUSSION

The relative growth of the chickens fed the diet diluted with 2% of cellulose was 92 to 101% of the controls (Table 2 and 3). When the diets contain 2% guar gum, the relative growth of the chickens was 41 to 67.4% of the controls. The depression in growth was probably not due to the dilution of the energy content of the diet by the addition of 2% gum because the addition of 2% cellulose which also diluted the energy of the diet to the same extent, caused little or no growth depression. The addition of the following polysaccharides at a level of 2% to the chicken diets caused a definite depression in growth of chickens: locust gum, gum tragacanth, gum karaya, dried okra, carrageenin, and psyllium husk (Tables 2 and 3). Pectin exerted a marked depression in chick growth at a level of 4%. At a level of 2%, growth of chickens on diets containing pectin, gum arabic, dextran, dextrin, gum ghatti, linseed mucilage, methylcellulose, carboxymethylcellulose, caramel, agar agar or kelp was of the same order as for control diets or those containing 2% cellulose.

As low as 1% guar gum in chicken diets caused a definite growth depression (Table 2). As the level of guar gum was decreased to 0.5 or 0.25%, the growth inhibition was also proportionately reduced. However, in case of locust gum, a definite growth inhibition of chicks occurred only at a 2% level.

Relation to composition. The variation in growth depressing effect of various polysaccharides prompted consideration of a possible common constituent responsible for this effect. Most of the data on the chemical constitution of these polysaccharides have been taken from the reviews of Smith and Montgomery (1959).

Guar gum and locust gum are neutral

[illegible] with the enzyme pectinase. When pectin and the enzyme were fed together, the growth inhibition was only partially overcome. The presence of methoxy groups in pectin was essential for its growth inhibition. This is borne out by the fact that polygalacturonic acid when fed at a level of 4% gave a relative growth of about 90% of the controls and the methoxy derivative of this (pectin) had a value of about 54%.

No information, as yet, is available about the exact sequence in which the various sugars are linked in the various polysaccharides. But it appears that as the frequency of branching increases in the molecule, the growth inhibitory properties also increase. Guar gum has D-galactose on every alternate D-mannose molecule in contrast to carob gum which has D-galactose on every 3rd or 4th mannose molecule of the straight chain part of the polysaccharide. Guar gum is more growth inhibitory than locust (carob) gum. Gum arabic has fewer branches and more of the component sugars are arranged as a straight chain. It has little growth inhibitory properties for chicks. In contrast to this, gum tragacanth has many more sugars branching from the straight chain of the polysaccharide and has definite growth inhibitory properties.

The ratio of feed intake/gain for controls and the diet containing 2% guar gum was of the same order (Table 2), but tended to be higher for okra, carrageenin, linseed mucilage, psyllium husk and carboxymethylcellulose (Table 3). Not all of these substances were growth inhibitory.

In general, the pancreas weight per 100 gm. body weight (Table 4) tended to be higher for those polysaccharides which had a growth inhibitory property. Chickens fed psyllium husk had an accumulation of material in the proventriculus which gave it an abnormal appearance of a sausage similar to ones observed by O'Dell *et al.* (1959). The incidence of this gross abnormality was only in chickens fed growth-inhibitory polysaccharides but was never as serious as for psyllium husk.

SUMMARY

Several naturally occurring polysaccharides depressed the growth of chickens when fed in a nutritionally balanced diet containing soybean meal, cereals, and fish meal. The growth of chickens was inhibited about 25% to 30% by the inclusion of guar gum, locust gum, gum tragacanth, gum karaya, or carrageenin at levels of 2%; or 4% of pectin in their diets. Dried okra or psyllium husk caused a depression in growth of about 15% at a level of 2% inclusion, and cellulose, methylcellulose, carboxymethylcellulose, dextrin, dextran, linseed mucilage, caramel, gum ghatti, agar agar and kelp appeared to be without any deleterious properties. The growth depressing properties of pectin and guar gum were overcome by their treatment with enzymes capable of hydrolyzing them, namely, pectinase and cellulase or a preparation from the sprouted guar beans.

ACKNOWLEDGMENTS

The authors wish to thank Stein, Hall and Co., Inc., 285 Madison Ave., New York 17, N.Y.; Dr. M. A. Joslyn, Department of Food Science, University of California, Berkeley, California; and Miles Chemical Co., Clifton, N.J., for donating some of the materials used in this study.

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A COMPARISON OF THE WEIGHT OF THE INTESTINE WITH THE BODY AND KIDNEY WEIGHTS IN RATS WHICH WERE FED ARTIFICIAL, UNBALANCED DIETS [1]

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It would seem reasonable to expect some alteration of the intestine when the physiologic load is increased, just as the heart and the kidney hypertrophy when more work is thrown upon those organs. For testing that hypothesis a group of animals were fed unbalanced, markedly different diets, and the intestine studied for changes, post-mortem. It was essential, however, that the diets employed be nutritionally adequate; otherwise the study would have been an investigation in malnutrition or starvation. The usual signs of health such as normal growth rate, smoothness of coat, brightness of eyes, fertility, and physical activity were noted. The work performed by the intestine has been divided into two phases — digestion and absorption. Anatomically the intestine is divided into two parts, large and small, but the work is shared jointly although not equally by the two parts. Theoretically a diet may affect structurally the whole intestine, or one of its parts.

Experimental studies on the effect of diet upon the intestine of fish, amphibia, and birds have been reported by Yung (1883, '09), Gulland (1898), Lonnberg ('02), Weiss ('01), Houssay ('02), Babàk ('06), Roux ('06), Schepelman ('06), and Elven ('28). These studies sprung primarily from the

[1] This investigation was supported by a grant from the Faculty Research Committee of the University of Pennsylvania.

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observations of de Cuvier and Milne-Edwards that the intestine was longer in herbivorous than in carnivorous animals. In addition, search for proof for Lamarck's theory of "use and disuse" was stimulating morphological investigation.

Another interest in the problem developed later when advances in the medical sciences acted as a stimulus to research in the relationship of diet and growth. In addition, the establishment of the value and importance of vitamins in the diet was of the greatest assistance in interpreting the conflicting findings in earlier investigations.

Wetzel ('28) studied the relation of the weight and length of the intestine to the body in rats which had been fed plant; meat, and mixed diets. The plant and mixed diets were fed to three, the meat diet to 7 rats. The plant diet consisted of wheat (sometimes corn) supplemented occasionally with oats, rye, barley, fresh fruits, nuts, and germinated peas. The meat diet consisted of raw beef alternated with raw horse meat, including the bones; occasionally raw frog muscle was supplied. Fresh milk was given daily. The mixed diet was a combination of the meat and plant diets. He found that the large intestine was heavier on the plant diet than on either the meat or the mixed diet, while the small intestine was lighter than on either of the other two diets. In the rats on the mixed diet both the small and large intestine were intermediate in weight.

Addis ('32) reported measurements on the intestine of rats which had been fed a concentrated diet or a contrasting bulky diet which contained 40% dry alfalfa and 10% dry agar by weight, for 44 weeks. He found a 48% increase in the weight of the large intestine, and a 10% increase in the weight of the small intestine on the bulky compared with the weight on the concentrated diet. These percentages were based on the area of the body surface. The reported increase on the bulky diet is, however, not to be interpreted as a real increase because the body surface on the bulky diet was considerably less than the body surface on the concentrated diet.

Wierda ('42) reported that rats which had been fed diets containing raw liver had a longer small intestine than rats which were fed a meat-free diet. However, rats which were fed raw beef or cooked dried beef showed no significant increase in the length of the small intestine. It was also reported that the length of the colon was not increased by the addition of meat to the diet. Rats which had been fed a diet containing from 10 to 30% finely ground agar showed no increase in the length of the small intestine; however, the caecum and colon were definitely longer than in the concentrated diet rats. The surface area of both small (excluding the villi) and large intestine was greater on the agar and meat diet than on the concentrated diet but the surface of the large intestine was definitely greater on the agar diet. The mucosa of the small intestine showed a definite response to the meat diet, the villi being large, high, wide and thick, and differing in these respects from the villi on the balanced concentrated diet.

Localized overgrowths of the wall of the colon were found in rats on a high-fat diet (Wierda, '43). The great size of the overgrowths which assumed the shape of diverticula indicated the existence of an exceedingly high growth potentiality. A similar finding was reported by Carlson and Hoelzel ('49) using a bulky diet.

MATERIALS AND METHODS

In these experiments 50 albino rats, including an equal number of each sex (Wistar strain) were maintained for 44 weeks on 4 different diets beginning at the time of weaning (see table 1). The animals were housed in screen-bottom cages and both food and water were kept continuously before the animals. The agar was finely ground, so that neither it nor any of the other ingredients could be separated from the diet by the rats. The salt was a modification of the Osborne-Mendel salt mixture (Wesson, '32). The rats used were the offspring of 10 matings, and each litter was spread over the 4 dietary groups as far and as evenly as possible so that

any hereditary differences would be distributed equally. At the end of the feeding period the rats were deprived of food for 24 hours before they were asphyxiated with coal-gas and autopsied. Each rat was weighed and its intestine carefully detached, slit open, and washed free from its contents. It was

TABLE 1

Average of measurments, with probable error, of 50 rats after 44 weeks of experimental feeding

NO. OF ANIMALS	COMPOSITION OF DIET		GROSS BODY WEIGHT	WEIGHT OF TOTAL FAT	WEIGHT OF SMALL INTESTINE	WEIGHT OF LARGE INTESTINE
14	Balanced		257 gm	16 gm	3.21 gm	1.83 gm
	Concentrated		± 10	± 1.9	± 0.08	± 0.04
	Casein	18%				
	Lard	23				
	Cornstarch	53				
	Salt mixture	4				
12	Bulky		261 gm	29 gm	3.76 gm	3.31 gm
	Casein	18%	± 11	± 1.4	± 0.21	± 0.17
	Lard	23				
	Cornstarch	23				
	Agar	30				
	Salt mixture	4				
12	Carbohydrate		268 gm	29 gm	4.26 gm	1.85 gm
	Casein	14%	± 12	± 6.8	± 0.24	± 0.06
	Cornstarch	83				
	Salt mixture	4				
12	Fat		278 gm	41 gm	3.86 gm	1.74 gm
	Casein	25%	± 11	± 5.2	± 0.15	± 0.07
	Lard	69				
	Salt mixture	6				

The diet of each rat was supplemented daily with 5 drops of cod liver oil and 0.4 gm of dry yeast.

then dried lightly with filter paper, placed in a stoppered weighing bottle, and weighed. The kidney was also carefully removed, dried by blotting with filter paper, and weighed in a stoppered weighing bottle. Finally the whole carcass was analyzed for fat using a modification of the Rose-Gottlieb

method for fat determination. The method consisted of saponification of each rat separately with potassium hydroxide, after which the volume was brought up to two liters, using a calibrated volumetric flask. An aliquot (10 cm^3) was transferred to a Röhrig tube and the fat separated by washing three times with 25 and 15 cm^3 of ether and petroleum ether. The combined washings were then freed from ether by evaporation on a steam bath to a constant weight. From this value the total amount of fat in the rat was calculated.

RESULTS

The averages of the measurements taken are shown in table 1. The average body weight at autopsy was nearly the same on the 4 diets. The greatest variation was between the balanced concentrated diet (257 gm) and the fat diet (278 gm). The gross weight on the bulky and concentrated diets was intermediate — 261 gm on the bulky diet and 268 gm on the carbohydrate diet. When the significance of these differences was tested statistically by the method of the probable error of the difference of the mean, it was found that there was no significant difference in gross weight. The probability was only equal that a difference in weight existed between the rats on the balanced concentrated diet that weighed the least and the fat diet rats that weighed the most.

The average amount of fat in the body (cellular and extracellular) at autopsy was least on the balance concentrated diet (16 gm) and greatest on the fat diet (41 gm), while on both the bulky and the carbohydrate diets the amount was 29 gm. When these figures were tested for validity by the method of the probable error of the difference of the mean it was found that it was a practical certainty that the fat diet rats possessed a greater amount of fat than the balanced concentrated diet rats. There was no significance to the difference in the amount of fat in the rats on the other diets.

The weight of the body of the rats, exclusive of fat, ranged less widely than the gross weight. The range of the net weight was from 241 gm on the balanced concentrated diet

to 232 gm on the bulky diet. In body tissues which were not saponifiable there was a spread of only 4% among the 4 groups.

The small intestine was heaviest on the carbohydrate diet, and lightest on the balanced concentrated diet. The difference in weight was 32%. The weight of the small intestine was on the average nearly the same on the fat diet and the bulky diet, and was not significantly less than on the carbohydrate diet, nor greater than on the balanced concentrated diet. The average weight of the small intestine on the 4 diets was 3.77 gm. On the balanced concentrated diet the weight of the small intestine was 17% less than the average of all 4 dietary groups; on the carbohydrate diet it was 11% greater; on the fat diet it was 2% greater, and on the bulky diet it was very nearly identical with the average. When these differences were checked by the probable error of the difference of the mean it was found that the probability was high that the small intestine was heavier on the carbohydrate than on the balanced concentrated diet. The probability was low for a real difference in weight between the other groups.

The large intestine was considerably heavier on the bulky diet than on the three contrasting (concentrated) diets. When the absolute weights were compared, it was found that the large intestine was 78% heavier on the bulky diet than on the carbohydrate diet; 80% heavier than on the balanced concentrated diet; and 90% heavier than on the fat diet. The difference between the weight of the large intestine on the bulky diet and on the three contrasting diets was great enough to make it highly probable that the large intestine was heavier on the bulky than on any of the other diets. When this test for significance was applied to the differences in weight of the large intestine on the carbohydrate, balanced concentrated, and fat diets, it was found that the probability was low that a real difference existed.

The average weight of the kidneys was greatest on the carbohydrate diet and least on the bulky diet. The difference in weight between these two groups was 27%, a difference

which when tested statistically indicated a high probability of a real difference. The average weight of the kidneys on the balanced concentrated diet was 17% less than on the carbohydrate diet, and on the fat diet the kidneys weighed 11% less than on the carbohydrate diet. But the kidneys of the balanced concentrated, fat, and carbohydrate diet animals were not significantly different in weight.

Table 2 shows the average weight of the kidneys in grams, and the ratio of the weight of the small and large intestine to the kidney weight after feeding the experimental diets for 44 weeks. On the balanced concentrated diet the small intestine was 155% of the weight of the kidneys; on the bulky

TABLE 2

The average weight of the kidneys in grams, with probable error; and the ratio of the weight of the small and large intestine to kidney weight after feeding the experimental diets for 44 weeks

TYPE OF DIET	KIDNEY WEIGHT	SMALL INTESTINE	LARGE INTESTINE
Balanced concentrated	2.07 ± 0.07	1.55	0.88
Bulky	1.91 ± 0.08	1.96	1.73
Carbohydrate	2.44 ± 0.10	1.74	0.75
Fat	2.19 ± 0.09	1.76	0.79

diet 196%; and on the carbohydrate and fat diet 174% and 176% respectively. In terms of kidney weight the small intestine was on the average 26% heavier on the bulky diet than on the balanced concentrated diet; and 12% heavier than on either the carbohydrate or fat diet.

The large intestine on the bulky diet was 173% heavier than the kidneys; on the balanced concentrated diet 88% heavier; on the carbohydrate diet 75% heavier; and on the fat diet 79% heavier. In terms of kidney weight the large intestine was 96% heavier on the bulky than on the balanced concentrated diet; 130% heavier than on the carbohydrate diet; and 118% heavier than on the fat diet.

DISCUSSION

The purpose of this experiment was to determine the effect of indigestible material in the diet upon the weight of the small and large intestine. Four diets were employed, one of which was a bulky diet containing a relatively large amount of agar — a poorly digestible substance, and three were contrasting, concentrated diets which contained no such ingredient but varied in respect to materials furnishing the calories. Of the three concentrated diets the fat diet had the highest caloric value — 7.21 calories per gram; the carbohydrate diet 3.88; and the balanced concentrated and bulky diets each had 4.91. To satisfy the caloric requirement it was necessary for the rats on the bulky diet to eat 4 times more material than the rats on the fat diet because dry agar is very bulky and has a low caloric value. Furthermore after the agar becomes wet it swells so that actually the intestine of the bulky diet animals handled approximately 10 times more material than the intestine of the rats on the fat diet. The carbohydrate and balanced concentrated diets did not swell upon wetting. Nevertheless, 4.5 times more material was handled by the intestine on the carbohydrate, and 5 times more on the balanced concentrated than on the fat diet. Wetzel ('28) did not calculate the relative amount of material handled by the intestine of his experimental animals, but Addis ('32) estimated that the intestine of his bulky diet rats handled 4 times more material (by volume) than the intestine of the "smooth" (concentrated) diet rats. Wetzel dealt particularly with the problem of the effect of meat, and of plant diets upon the weight and length of segments of the intestine, as well as the whole tract.

Both Wetzel ('28) and Addis ('32) encountered difficulty in interpreting the observed weight of the intestine on the contrasted diets because of the difference in body size of the rats. In Wetzel's experiments the difference was not great, but he believed that it accounted for the relatively heavier small intestine on the "plant" diet compared with the "flesh" diet. In the experiments of Addis the body weight of the

bulky diet rats was not much more than half that of the "smooth" (concentrated) diet animals, consequently no direct comparison of the weight of the intestine was made, but its weight was compared with the body surface. (Using the formula — body surface $= 11.3 \times 0.666$ body weight.)

In the present experiments the rats weighed approximately the same, at autopsy, on the 4 diets. This fact indicates that agar can compose as much as 30% of the diet of the rat without a resultant decrease in the growth rate of the body compared with the artificial diets containing no bulky ingredient. The fat determination shows that only the fat diet rats contained significantly more fat than the bodies of the leanest, i.e., the concentrated diet rats. The weight of the non-fat part of the rats was nearly the same on each of the diets, a fact which shows further that the potentiality for growth was not inhibited by any of the diets.

At autopsy the rats which had been fed the carbohydrate diet had a small intestine which exceeded the average weight on the other diets, and was also significantly heavier (statistically) than in the rats which had been fed the balanced concentrated diet. It is not clearly seen why there is this contrast in effect between these two diets. According to the theory of Roux ('06) a concentrated diet, rich in easily digestible substances, is capable of stimulating the growth of the small intestine, thereby enabling a greater and more rapid digestion and absorption of the food, but in these experiments the bulky diet (30% agar) rats showed a heavier small intestine, at autopsy, than the rats which had been fed a concentrated balanced diet. Such a finding is plainly not in accord with Roux's theory — for the balanced concentrated diet is clearly more easily digested than the bulky diet. According to Wetzel ('28) and Wierda ('42) the feeding of a meat diet or a diet containing meat resulted in a longer and heavier small intestine in rats than a plant diet, or a diet containing casein as the source of protein. Addis ('32), however, reported finding an 8% increase in the weight of the small intestine on a bulky diet, but since this calculation is

based on the body surface of rats which weighed only half as much as the controls, there is reason to doubt the existence of a real increase.

The weight of the large intestine at autopsy clearly shows that hypertrophy has occurred on the bulky diet. For although the body size was nearly the same as on the concentrated diets, the large intestine was 78% heavier than on the carbohydrate diet; 80% heavier than on the balanced concentrated diet; and 90% heavier than on the fat diet. These values are considerably higher than Addis ('32) found. He reported only a 48% increase in weight for the large intestine but the contrast in his diets was less than in these experiments and, in addition, his bulky diet animals were only half the size of those on the concentrated diet.

The weights of the kidneys were compared with the weight of the small and large intestine because the kidneys have been used frequently as a criterion for enlargement of other organs, although it has been shown by several investigators that the coefficient of correlation of kidney weight with body weight is very high. In the rat on a stock diet values of 0.96 and 0.95 were reported for males and females respectively (Webster, Liljegren and Zimmers, '47). In terms of kidney weight the small intestine was found to be heaviest on the bulky diet, exceeding the weight on the concentrated balanced diet by 26% and both the carbohydrate and fat diets by 12%. The large intestine was likewise heaviest on the bulky diet in terms of kidney weight, exceeding the weight on the balanced concentrated diet by 96%, and on carbohydrate and fat diet by 130 and 118% respectively. The observed weight of the large intestine after 44 weeks of feeding a bulky diet indicates an enlargement of approximately the same magnitude as Flint ('12) observed in dogs from whom 50 to 70% of the small intestine had been removed surgically. No gross changes in the large intestine, such as diverticula, were observed in any of these rats but there was a marked increase in caliber (diameter) as well as in the thickness of the wall.

In aged rats hypertrophy of the colon through the development of diverticula has been reported by Wierda ('43) and Carlson and Hoelzel ('49).

SUMMARY

A group of 50 albino rats were fed an artificial unbalanced diet beginning at the time of weaning and continuing for 44 weeks, to determine the effect upon the weight of the small and large intestine. The animals were asphyxiated with coal-gas after having been starved for 24 hours. The small and large intestine and the kidneys were then carefully isolated, trimmed free from attached tissue, and weighed using glass stoppered weighing bottles to prevent dehydration. The carcass was saponified and the amount of fat in it was determined by a modification of Rose-Gottlieb method for fat determination. The weight of the small and large intestine on the 4 diets were then compared with each other and with the body (gross, and minus fat) and kidney weight.

The gross weight of the rats was found to be not significantly different on the 4 diets; however, those which had received the fat diet possessed a significantly greater amount of fat than those which had received the balanced concentrated diet. The net weight of the body (gross weight minus fat) was even more nearly the same on the 4 diets than the gross weight.

The small intestine was found to be heaviest on the carbohydrate diet and lightest on the balanced concentrated diet. This difference was great enough to be statistically significant; the average difference was 32%. The small intestine was intermediate in weight between these two extremes on the fat and bulky diets. These differences in weight cannot be accounted for on the basis of the concentration of the diet. The heavier weight of the intestine on the carbohydrate diet may be due to the ease with which the diet is digested, and the rapidity with which it moves through the small intestine, but the balanced concentrated diet differs hardly enough from the carbohydrate diet to account for the lightness in weight of

the small intestine on the balanced concentrated diet. The large intestine was significantly heavier in the rats which had been fed the bulky diet than in those which had received the concentrated diets. The average weight was 78% greater than on the carbohydrate diet; 80% greater than on the balanced concentrated diet; and 90% greater than on the fat diet.

In terms of kidney weight the small intestine was on the average 26% heavier on the bulky diet than on the balanced concentrated diet, and 12% heavier than on either the carbohydrate or fat diet. The large intestine was, in terms of kidney weight, 96% heavier on the bulky diet than on the balanced concentrated diet; 130% heavier than on the carbohydrate diet; and 118% heavier than on the fat diet. The increased weight of the colon was not due to diverticula, but primarily to an increase in diameter, and a thickening of the wall.

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MEASUREMENTS AND OBSERVATIONS UPON THE INTESTINE OF RATS FED UNBALANCED AND SUPPLEMENTED DIETS[1]

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ONE PLATE (FIFTEEN FIGURES)

Two factors are supposed to operate in altering the generic nature of the digestive tract. These factors are the diet and the secretion of the anterior pituitary gland. The experiments to be described deal with the effect of certain diets upon the length and the amount of surface of both the small and large intestine, and upon the villi of the small intestine.

Experimental studies to determine the ability of the diet to alter the morphology of the digestive tract have been made by Yung (1883, '09), Gulland (1898), Lonnberg ('02), Weiss ('01), Houssay ('02), Babak ('06), Roux ('06), Schepelman ('06), and others. In addition to these contributors, there is the more recent work of Elven ('28) on the frog, and Wetzel ('28) and Addis ('32), both of whom used the rat. At the present time the contention that the diet of a rat can increase or decrease the length of the intestine or the surface area of the intestine rests upon the experiments of Wetzel and of Addis. Wetzel ('28) made the following statement in his conclusions: "On a flesh diet, the small intestine of the rat becomes longer compared to the large intestine, while the large intestine is short. Inversely, the large intestine is compara-

[1] The work reported in this paper was done under the direction of the late Prof. A. T. Kerr and was submitted to the graduate faculty of Cornell University, Ithaca, N. Y., as a portion of a thesis in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Amer. J. Anat., 1942, 70: 433-53

tively longer in rats on raw plant diet, ... the small intestine is relatively shorter." Also, "On the basis of body length the large intestine is longer in the plant fed rats, the length of the small intestine, on the other hand, is less than in the flesh fed rats." Addis ('32) reported a 10% increase in the length of the small intestine, and a 34% increase in the length of the colon in rats which were fed a diet consisting of 40% dry alfalfa and 10% agar for a period of 44 days, when compared with rats which were fed a "smooth" diet. These figures, however, are not direct percentage increases in length but are based on the body length measurement. And since the experimental animals were only 50% as heavy (body weight) as the control group of rats, the body length of the two groups must also have varied greatly, so that the increase in the length of the intestine as expressed was definitely a "relative" increase, and measured in rats which were undernourished.

Riddle and Flemion ('28) reported that in doves the intestine was definitely longer in the female than in the male, and that they believed this difference was associated with the larger size of the anterior pituitary gland of the female. Other references are found in the literature in which hypersecretion of the anterior pituitary is associated with splanchnomegaly (Barker, '18). But no experimental evidence has been presented to show that the anterior lobe secretion has a specific effect upon the growth of the intestine, that it causes a real increase in the length or the circumference of the intestine, or increases the surface of the intestine.

MATERIALS AND METHODS

The rats used in these experiments were of the Wistar strain. They were housed in galvanized iron cages, with wire bottoms. The composition of the diets which were used is shown in the tables with the measurements. A supply of food was constantly available to the rats, and likewise drinking water. The rats were deprived of food for 24 hours before they were killed with coal-gas and autopsied. The intestine

was measured for length after it had been detached from the mesentery and this was done immediately after death. In some cases, as is indicated in the tables, the intestine was measured for length before it was detached from the mesentery by placing a moist thread along the course of the intestine, and then ascertaining the length of the thread. The detached intestine was measured for length while it was suspended at one end and hanging either freely or with a small weight attached to the lower end. The area of the surface of the intestine was measured by slitting open the intestine, washing, and submerging in 2% potassium dichromate solution for about 12 hours. This solution fixes the intestine slightly but does not cause contraction. The intestine was then cut into pieces of a convenient length (10 to 20 cm.) and carefully pinned to a cork-board so that each segment was stretched as equally as possible in the transverse and lengthwise direction. The width of each segment was measured at intervals of approximately 8 mm. with a caliper.

Photographs were taken of the mucosal surface of the intestine at the pylorus, at the caecum, and half-way between these two boundaries, of the animals kept on the diets shown in table 1. A Tessar lens was used, and the source of light was a carbon arc lamp.

All the diets were composed of basic, pure, food substances — casein, lard and corn starch. Each diet contained 4% of Osborne and Mendel salt mixture with the exception of the fat diet, which contained 6%, and the carbohydrate diet, which contained 3.2%. Each rat received daily 5 drops of cod liver oil and 0.4 gm. of dried yeast.

RESULTS OF MEASUREMENTS

Table 1 presents the measurements which were taken at autopsy after the rats had been kept on the diet as indicated, for a period of 30 weeks. If the group which was fed the balanced concentrated diet is taken to be the control group, then it can be said that the small intestine is 9.8% longer in the rats which were fed a diet of 10% agar agar. The small

intestine of the rats which were fed the diet supplemented with liver was 12.5% longer than in the rats which received the balanced concentrated diet. The small intestine of the carbohydrate diet rats was 5% longer, and on the fat diet it was 1.8% longer than the small intestine of the rats which were fed the balanced concentrated diet. Only in the case of the diet supplemented with liver is the increase great enough

TABLE 1

Average of measurements of sixty rats after 30 weeks of experimental feeding.

TYPE OF DIET	COMPOSITION OF DIET[1]		BODY WEIGHT	BODY LENGTH	LENGTH OF SMALL INT.	LENGTH OF COLON
Balanced concentrated diet	Casein	18	224 gm. ± 15	21.5 cm. ± 0.7	111.8 cm. ± 4.2	13.6 cm. ± 0.9
	Lard	23				
	Sucrose	53				
Bulky diet	Casein	18	281 gm. ± 19	22.1 cm. ± 0.9	122.7 cm. ± 7.3	16.5 cm. ± 0.9
	Lard	23				
	Sucrose	43				
	Agar agar	10				
Balanced concentrated diet + liver[2]	Casein	15	328 gm. ± 32	22.5 cm. ± 0.7	125.7 cm. ± 6.9	15.2 cm. ± 0.7
	Lard	20				
	Sucrose	49				
Carbohydrate diet	Casein	14	245 gm. ± 20	21.0 cm. ± 0.5	117.3 cm. ± 5.7	14.1 cm. ± 0.8
	Sucrose	83				
Fat diet	Casein	25	197 gm. ± 15	21.2 cm. ± 0.6	113.8 cm. ± 7.0	13.2 cm. ± 0.5
	Lard	69				

[1] Minerals and vitamins were supplied as stated in body of paper.
[2] Five grams to each rat per day.

to be significant. The chance of a real difference occurring in these two groups is better than 22 to 1. On the other diets there is no significant change.

In regard to the colon, table 1 shows that it is on the average 2.9 cm. longer in the rats on the agar diet than in the rats on the concentrated balanced diet. The probable error of the difference of these two groups is 0.88 and since this is less than one-third of the difference of the average, the chance is about 22 to 1 that a real difference occurs. The colon in the rats

which received the liver is on the average 1.6 cm. longer than in the balanced concentrated diet group, but statistically there is no real difference in the colon of these two groups. There is also no difference between the length of the colon in the carbohydrate diet rats and the high fat diet rats.

The body weight of the rats which received the 10% agar diet is on the average 57 gm. greater than that of the rats on the concentrated balanced diet. This difference compared to the probable error of the difference of the means, which is 16.3, indicates a better than 22 to 1 chance of a real difference occurring. The rats which received the diet supplemented with liver weighed on the average 104 gm. more than the concentrated diet group of rats. Thus, it is practically a certainty that the rats which received the agar diet are heavier in body weight, since the probable error of the difference is 24, or less than one-fourth of the difference of the means. The rats which received the carbohydrate or the fat diet did not differ appreciably in respect to body weight from the rats which received the concentrated balanced diet.

The body length of the rats (snout-anus length) is fairly uniform. The greatest difference is between the liver supplemented diet group and the carbohydrate diet group. Between these two groups there is a 4½ to 1 chance of a real difference occurring. None of the groups differ widely enough from the balanced concentrated diet group to indicate a real difference in body length.

The measurements presented in table 2 were made to determine whether meat would alter the length of the small intestine or the colon compared to a diet in which casein was the sole source of protein. Liver and steak, in both the raw and cooked state, were used. The diets were continued for 36 weeks, beginning at the time of weaning (28 days) and ending with autopsy. The intestine of these animals was measured for length while it was suspended freely, in contrast to the method used in the animals included in table 1, when a small weight of approximately 4 gm. was attached to the

lower end while the measurement was taken. Rats of both sexes were used (table 2).

The length of the small intestine and the colon of the animals which received meat was not significantly greater or smaller than in the rats which received no meat but casein alone as a source of protein.

TABLE 2

Average of measurements of fifty two rats after 36 weeks of experimental feeding.

TYPE OF DIET	COMPOSITION OF DIET		BODY WEIGHT	BODY LENGTH	LENGTH OF SMALL INT.	LENGTH OF COLON
Balanced concentrated diet	Casein	25	307 gm. ± 26	22.7 cm. ± 0.8	119.3 cm. ± 4.9	15.2 cm. ± 0.7
	Lard	15				
	Sucrose	46				
Balanced concentrated diet + raw liver	Casein	15	344 gm. ± 19	23.0 cm. ± 1.1	115.7 cm. ± 6.8	14.7 cm. ± 1.1
	Lard	15				
	Sucrose	44				
	Raw liver	33				
Balanced concentrated diet + dried liver	Casein	15	358 gm. ± 64	22.8 cm. ± 1.5	124.2 cm. ± 12.1	16.4 cm. ± 1.3
	Lard	15				
	Sucrose	44				
	Dried liver	12				
Balanced concentrated diet + dried steak	Casein	15	340 gm. ± 70	22.6 cm. ± 1.1	119.7 cm. ± 6.5	16.0 cm. ± 1.0
	Lard	15				
	Sucrose	44				
	Dried steak	12				
Balanced concentrated diet + raw steak	Casein	15	267 gm. ± 30	21.5 cm. ± 0.9	118.3 cm. ± 6.8	16.3 cm. ± 1.0
	Lard	15				
	Sucrose	44				
	Raw steak	33				

The rats which received the diet supplemented with raw steak were on the average definitely lighter in body weight than the rats which received the cooked and dried steak or raw or cooked liver. The average weight of these (raw steak) rats was even less than the average weight of those which received no meat. When the body weight of any two groups of these rats (table 2) was compared and checked by the statistical method of the probable error of the differences it

was found that the probability of a real difference occurring was very slight. The difference in body length of the meat diet rats and the meat-free diet rats was not significant.

Table 3 shows the measurements which were made on two groups of male rats. One group was fed a balanced concentrated diet, and the other group was fed a diet which consisted of nearly 30% cooked and dried liver and steak. The rats were placed on these diets at the time of weaning (28

TABLE 3

Average of body and intestine measurements of twenty-four male rats, comparing effect of casein and meat after 36 weeks of feeding.

MEASUREMENT	CONCENTRATED BALANCED DIET *	CONCENTRATED DIET + RAW MEAT *	DIF. MEAN / P. E. OF DIFF.	PROBABILITY OF REAL DIFFERENCE
Body weight	334 gm. ± 20	427 gm. ± 25	2.8	Favorable
Body length	23.3 cm. ± 0.4	24.3 cm. ± 0.8	1.0	Equal
Small intestine (in situ)	85.9 cm. ± 1.4	96.1 cm. ± 6.3	1.5	Equal
Small intestine (detached)	120.2 cm. ± 6.0	128.2 cm. ± 5.5	0.9	Equal
Colon (in situ)	14.2 cm. ± 0.7	15.8 cm. ± 0.7	1.5	Equal
Colon (detached)	15.8 cm. ± 0.6	17.0 cm. ± 0.7	1.2	Equal
Cæcum	6.8 cm. ± 0.3	6.9 cm. ± 1.0	0.0	Improbable
	*Casein 25 Lard 15 Sucrose 46	*Casein 16 Lard 15 Sucrose 44 Liver and steak 33		

days of age) and the diet was continued for 36 weeks. The measurements and the probable error of each are given in columns two and three. In the fourth column the ratio of the difference of the means to the probable error of the difference is given. In the last column the probability of a real difference occurring is stated.

If the group of rats which received the balanced concentrated diet is considered to be the control group of animals, and the meat diet group is considered as being the experimental group, it can be stated that the addition of meat to the diet resulted in an increase in body weight over that of the control rats. The rats which received the diet supple-

TABLE 4

Measurements of twenty-four rats which were fed a concentrated balanced diet and an agar diet for 40 weeks.

MEASUREMENT	CONCENTRATED BALANCED DIET *	BULKY DIET *	DIF. MEAN / P. E. OF DIFF.	PROBABILITY OF REAL DIFFERENCE
Body weight	203 gm. ± 21	245 gm. ± 20	1.1	Equal
Body length	20.2 cm. ± 0.8	21.8 cm. ± 0.9	1.3	Equal
Small intestine (in situ)	99.8 cm. ± 4.7	113.0 cm. ± 2.8	2.4	Favorable
Small intestine (detached)	120.7 cm. ± 5.4	128.3 cm. ± 5.1	1.0	Equal
Colon (in situ)	14.7 cm. ± 0.6	18.8 cm. ± 0.6	4.2	Practical certainty
Colon (detached)	16.0 cm. ± 0.4	20.4 cm. ± 0.5	6.3	Practical certainty
Cæcum (in situ)	5.3 cm. ± 0.3	7.5 cm. ± 0.8	2.3	Favorable
Cæcum (detached)	5.5 cm. ± 0.4	8.0 cm. ± 0.9	2.5	Favorable
	*Casein 18 Lard 23 Sucrose 53	*Casein 18 Lard 23 Sucrose 23 Agar 30		

mented with meat, even though the average body weight was 93 gm. heavier and the probability is high that a real difference in weight existed, did not show a real difference in length of the small intestine, the colon, or the cæcum when checked by the statistical method.

Table 4 shows the measurements which were taken on two groups of rats which were also litter mates. The one group

was fed a balanced concentrated diet for 40 weeks beginning at weaning, and the other group was fed a diet in which agar (very finely ground) was substituted for a large part of the sucrose (30 parts of agar for 30 parts of sucrose). The animals were deprived of food for 24 hours before they were sacrificed and measured. The weight is expressed in grams and the linear measurements in centimeters; the probable error of the measurements is also given. In the third column the ratio of the difference of the means to the probable error of the difference is given. In the last column the degree of probability of a real difference occurring is stated. Considering the group which received the concentrated balanced diet as a control group, a comparison shows that the group which received the 30% agar (approximately) diet were not definitely different in body weight or body length. The chance of the small intestine being longer in the agar diet rats is 4½ to 1 when measured in situ, but only 1 to 1 when compared after detachment and measured while suspended. The colon is definitely longer in the agar diet rats than in the control group of rats, whether measured in situ, or after detachment. The chances of a real difference occurring are 142 to 1. The caecum was also longer in the rats which received the agar diet than in the control group. The chances of a real difference occurring are 4½ to 1. Direct comparison of the length of the intestine in these two groups of rats shows that the small intestine is on the average 13% longer in the rats which were fed agar than in the control animals, when the measurement was taken while the intestine was in situ whereas it was 6.3% longer when measured after detachment. The colon was 27.8% longer in the agar diet animals than in the control animals when measured in situ, and 27.5% longer when measured after it was detached. The caecum was 41% longer when measured in situ, and 45% longer when detached, in the rats which were fed agar than in the control group.

Table 5 shows the surface area of the body, the small intestine, and the colon of thirty-six rats which were fed a concentrated balanced diet, a 30% agar diet, and a diet con-

sisting of 30% raw meat. Each measurement is an average of the measurement of twelve rats. The gross body weight is also given, and the length of the small intestine and the colon. The area of the surface of the body was calculated by using the formula of Lee and Clark ('29) which contains the nutritional correction factor. The method used to measure the area of the intestine has been described. It does not include the area of the villi.

The measurements made on these rats show that after 40 weeks of feeding a diet composed of 30% agar the length of the small intestine was on the average 6% (8 cm.) longer

TABLE 5

Increase surface area of body, small intestine and colon of rats which were fed a conc. bal. diet, a 30% agar diet, and a diet containing 33% of meat for 40 weeks.

SURFACE AREA OF	CONCENTRATED BAL. DIET RATS	AGAR DIET RATS (30%)	MEAT DIET RATS (33%)
Body	293 sq. cm. (Bd. wt. 203 gm.)	317 sq. cm. (Bd. wt. 228 gm.)	399 sq. cm. (Bd. wt. 369 gm.)
Small int.	132 sq. cm. (L. = 120 cm.)	152 sq. cm. (L. = 128 cm.)	148 sq. cm. (L. = 124 cm.)
Colon (exclusive of caecum	33 sq. cm. (L. = 21 cm.)	56 sq. cm. (L. = 25 cm.)	42 sq. cm. (L. = 22 cm.)

than in those fed a balanced diet containing no roughage. Furthermore, the small intestine was 7% greater in circumference and had 15% more surface. The colon of these same animals was 19% longer, 42% greater in circumference, and had 69% more surface than the colon of the rats which were fed the concentrated diet.

The rats which received a diet consisting of 33% raw meat for 40 weeks had a small intestine which was on the average 3% longer, 8% greater in circumference, and had 12% more surface than those which were fed a concentrated balanced diet containing 25% protein. The colon of these same animals was 4.7% longer, 21% greater in circumference, and had 27%

more surface than the colon of the rats which were fed the concentrated balanced diet.

The length of the small intestine was not significantly different in the three groups (table 5), but the area of its surface was significantly greater after feeding the agar diet or the diet consisting of 33% meat than in those rats which received the concentrated balanced diet. The colon was significantly longer in the rats which were fed the agar diet than it was in either of the other two groups, and the area of the surface was also greater.

The rats which received the diet consisting of 33% raw meat have significantly more colon surface than those which received the concentrated balanced diet; the length of the colon was, however, not significantly greater.

The nature of the mucosal surface of the small intestine after 8 weeks of feeding of the diets described in table 1 is shown in plate 1. Three regions of the small intestine are shown: (1) the duodenum, just below the pyloric valve, (2) the lower region of the jejunum, and (3) the end of the ileum, just above the ileo-caecal valve. The photographs are arranged so that each column represents a type of diet, and the top figure of each column portrays the duodenum, the middle figure the jejunum, and the lowest figure the ileum. Each figure is placed so that it shows a transverse view of the intestine. The first column shows the mucosa of the small intestine in a rat which was fed a high-fat, carbohydrate-free diet, column 2 the mucosa of a rat which was fed a high-carbohydrate, fat-free diet, column 3 the mucosa of a rat which was fed a diet supplemented with liver, column 4 the mucosa of a rat fed a diet consisting of 10% agar agar, and column 5 that of a rat fed a balanced concentrated diet (see table 1).

DISCUSSION

Three form- and growth-determining factors have been considered responsible for the final product of the growth process. These factors are: (1) Inheritance through the germ plasm, (2) growth as the result of functional stimulation, and (3) the effect of various physiological agents, such as the endocrine secretions. The post-natal effect of food upon the growth and differentiation of the digestive tract appears to be an example of the second of these factors. Measurements of the intestine such as have been made, upon animals which have been fed unbalanced diets for prolonged periods of time, should give some information concerning the effectiveness of the diet as a stimulus to the growth of the tract. During the experimental period of functional stimulation by the feeding of an unbalanced diet, the third factor is continuously working an effect upon the digestive tract. The genetic factor is also operating in these experiments. It almost certainly sets the limit beyond which an increase in size does not, or cannot extend.

A long account of the way in which the diet reacts upon the digestive tract and causes alterations is given by Wetzel ('28). In that publication he reviewed the opinions of Schepelmann, Babak, Elven, and Roux.

The classical example of the growth of an organ as the result of physiological stimulation is that of the kidney, which practically doubles its size when, following surgical removal of one kidney, the remaining one takes over the work of both. The intestine is also an organ capable of growth as the result of physiological stimulation, according to the work of Flint ('12). He found that in resection of the intestine of the dog there was a marked increase in the diameter of the remaining (functional) part of the intestine, which appeared to be twice the size of the intestine at the time of operation. No increase in length of the remaining part of the organ was found, but the villi and crypts were so greatly enlarged that the area of the surface of the original intestine was approximately restored. If there was no increase in length of the remaining part of the small intestine after resection of 50 to 70% of the original length, the probability might appear to be very slight that any increase in the length of the intestine of an intact animal would result from changes in the diet alone. In this case, the need for hypertrophy would be considerably less

than in the animal which had lost a large part of its intestine by resection. However, some investigators have reported that an increase in length does occur. Those investigators previously mentioned who were interested in the ability of the intestine to increase in length when the amount of work imposed on it was increased, have used in general two contrasting diets, one which is easily digestible, of high calorific value, and which leaves little indigestible residue, and a contrasting diet containing cellulose which is less easily digested, has a lower calorific value, and leaves a large amount of residue. It was believed that the easily digestible diet had a stimulating effect upon the small intestine, and the high residue diet a stimulating effect upon the colon.

The feeding of such contrasting diets has resulted in considerable variation in the size of the animal. In the case of Addis' experiments as high as 50% difference in body weight resulted. This difference in size of the contrasting groups has led to the expression of the length of the small and large intestine in terms of body length, or the length of the small intestine in terms of the colon (Wetzel), or the length of the colon in terms of the small intestine (Wetzel). It seems important enough to mention, however, that Wetzel's experiments included only three animals on a plant (high residue) diet and seven animals which were fed a meat diet. In his experiments the high residue consisted of wheat (or corn) and sugar beets supplemented occasionally with oats, rye, barley, fruits (such as fresh cherries, pears and oranges), nuts and germinated peas. The low residue (meat) diet consisted principally of raw beef, alternated frequently with raw horse meat, including the bones. Occasionally uncooked frog muscle was also given; fresh milk was a daily constituent of the diet. The rats differed markedly in weight at the time of autopsy, even on the same diet, and since his measurements were made after fixation in formalin, the intestine and colon length is approximately 30% less than when measured in the fresh state, as was the case in the experiments reported here.

When agar agar is used exclusively in the diet to give bulkiness and composes 30% of the diet by weight (dry), the intestine is subjected to a very large amount of material. However, there is no indication of any deleterious effect if one judges by the growth rate and by the absence of any recognizable pathology of the digestive tract. The mass of the content of the intestine on the 30% agar diet is approximately four times as great as on the concentrated balanced diet, due to the water absorbing power of the agar, and approximately six times as great as on the diet composed of casein and lard. However, the large amount of work placed upon the wall of the intestine by this bulky diet has no significant effect upon the absolute length of the small intestine.

The nature of the protein in the diet, whether casein or meat, had no effect upon the absolute length of the small intestine, even in the rats which received the raw steak, and in which the body weight was definitely less than that of the rats on the other meat diets, the small intestine was not definitely shorter. The measurements of the small intestine of the animals which were fed liver and cooked steak did not vary appreciably from the measurements of those which received casein. These comparisons show that the rat is in a sense unlike the tadpole, for in that animal crab meat resulted in a lengthening of the intestine, while beef-steak caused a shortening and widening (Babak, '06).

The colon was definitely longer in the rats which received the agar diet than in the rats which received the concentrated diet. The absolute increase was, however, not nearly so great as the 34% (relative) increase in length implies which was found by Addis ('32) on feeding a diet of 40% dry alfalfa and 10% agar agar. It seems worthy of mention that the bulky diet rats of Addis weighed approximately one-half as much as the smooth diet control animals and were suffering from malnutrition. Under such conditions, the factor of inanition would appear to enter the experiment and add stress to the interpretation. The length of the intestine may well increase

while the body growth is stationary, but the tone of the muscle of the intestine may also be decreased during inanition (Rowlands and Browning, '28) and account for part of the increase in length.

The observations of Jackson ('39) on the effect of inanition and subsequent refeeding upon the weight of the intestine show that the intestine of rats which had been kept at constant body weight for a period of 15 weeks weighed slightly less than the intestine of control rats of the same body size which were 3 weeks of age. The decrease in weight was, however, not statistically significant. Upon refeeding these stunted rats until the body weight had increased 100%, the intestine increased in weight, but weighed significantly less than that of normal rats of the same body weight. Although these measurements indicate no definite change in the dimensions of the intestine, they indicate a quantitative difference in response to inanition and to subsequent refeeding of the intestine and the body as a whole.

The measurements which were made upon the small intestine and colon to ascertain the area of these two parts of the digestive tract after feeding a concentrated balanced diet, a 30% agar diet, and a 33% meat diet indicate that these two parts of the tract hypertrophy relatively more in circumference than in length. The measurements (table 5) show that both the meat diet and the diet containing agar result in a greater (percentage) increase in circumference than in length, this being more marked in the case of the colon than the small intestine. The latter, it was found, had 12% more surface (on the average) in the rats which received the meat diet than in those which received a diet containing no meat, casein alone providing the source of the protein. The small intestine of the rats which received the 30% agar diet had 15% more surface, again excluding the surface of the villi, than those which received the concentrated balanced diet (table 5).

The colon surface was increased 27% in surface area in the meat diet rats, and 69% in the agar-containing diet rats

over the amount of surface found in those which received the concentrated balanced diet (table 5). This finding is at variance with that of Wetzel ('28) who believes that meat has no effect upon the colon. The increase in the surface of the small intestine and the colon of the rat is clearly seen on both the meat and the agar agar diet, and does not indicate a specific action of meat and a bulky diet upon either division of the digestive tract, which view is supported by Wetzel ('28).

The difference in the mucosal surface of the small intestine in rats which had been fed markedly different diets for 8 weeks is seen by comparing the photographs of plate 1. The composition of these diets is given in table 1. The top row, which shows the duodenum, shows the greatest range of variation in respect to the size and shape of the villi. The mucosa of a rat which received a normal concentrated diet is placed at the right (figs. 13, 14, 15). In the rat which was fed a diet supplemented with liver the villi are extremely long (figs. 7, 8 and 9), extending in some cases nearly two-thirds of the distance around the intestine. They are also higher, when measured from the base to their free border, and are very thin, delicate, and almost translucent (fig. 7).

In the rat which was fed a carbohydrate diet the villi of the duodenum (fig. 4) are very definitely longer, higher and thicker than on a normal concentrated diet (fig. 13) but they are characteristically different from those in the rat which received the liver supplemented diet. In the rat which was fed a diet consisting of 10% agar agar the villi of the duodenum (fig. 10) resemble those of the duodenum of the carbohydrate diet rat (fig. 4). They differ greatly from the villi of the other duodena (figs. 1, 7 and 13). The villi of the duodenum of the rat which was fed a fat diet (fig. 1) resemble those found in the one which was fed a normal concentrated diet (fig. 13).

In reference to the villi of the lower part of the jejunum (see middle horizontal row of figures of plate 1) it is to be noted that the variation in the villi is not as great as in the duodenum, yet the individuality seen most clearly in the [illegible] series throughout and also characterizes the jejunum.

The bottom row of figures, showing the mucosal surface of the ileum just before the ileo-caecal junction, reveals the same degree of similarities and differences on the different diets as is seen in the jejunum. Here again, the villi of the high-fat diet rat (fig. 3) resemble those of the rat which was fed a normal concentrated diet (fig. 15). The villi of the ileum of the rat which was fed a carbohydrate diet (fig. 6) resemble most closely those of the rat which was fed a diet supplemented with liver (fig. 9). In the ileum of the rat which was fed a fat diet (fig. 3) and a concentrated balanced diet (fig. 15), the openings of the mucous glands are rather plainly seen. Measurements of the villi were not made because of the technical difficulties, which are apparent from the photographs.

Bujard ('08) made a study of the intestinal villi of the rat under experimental dietary conditions. He reported changes in the size and shape, and concluded that the "variations are confined and limited to the second half of the ileum." The photographs of plate 1 do not confirm this finding. The greatest variation has been found to occur in the duodenum.

SUMMARY AND CONCLUSIONS

The effect of meat upon the length of the intestine can be summarized as follows:

1. The length of the small intestine is increased in rats which received 5 gm. of raw liver each day compared to the length in rats which received no meat but these rats were also definitely heavier in body weight.

2. Rats which received a diet composed of 33% raw meat or 12% dried meat showed no significant increase in the length of the small intestine, and the body weight was also not definitely greater.

3. The colon is not significantly longer in rats which have received meat.

The effect of agar agar upon the length of the intestine is as follows:

1. The length of the small intestine is not significantly altered by a diet containing either 10 or 30% agar agar compared to the length observed in rats which were fed a concentrated balanced diet, and the body weight of the bulky diet rats is as great as those which received the concentrated balanced diet.

2. The colon and caecum are definitely longer in the bulky diet rats than in the concentrated balanced diet rats.

The effect of meat and of agar agar upon the circumference and area of the intestine can be summarized as follows:

1. The surface area of the small intestine was 15% greater in the rats which were fed a 30% agar diet than in those rats which were fed the concentrated balanced diet.

2. The surface area of the colon (excluding caecum) was 69% greater in the 30% agar diet rats than in the concentrated diet rats.

3. The surface area of the small intestine was 12% greater in the meat (33%) diet rats than in the concentrated balanced diet rats.

4. The surface area of the colon was 27% greater in the meat diet rats than in the concentrated balanced diet.

5. Agar agar (30%) increases the area of the small and large intestine more than meat, and affects the colon more than the small intestine.

The morphology of the intestinal villi is altered by the diet, and the changes which are most clearly seen in the duodenum, are probably of a greater order than the changes in the length and the area of the small intestine.

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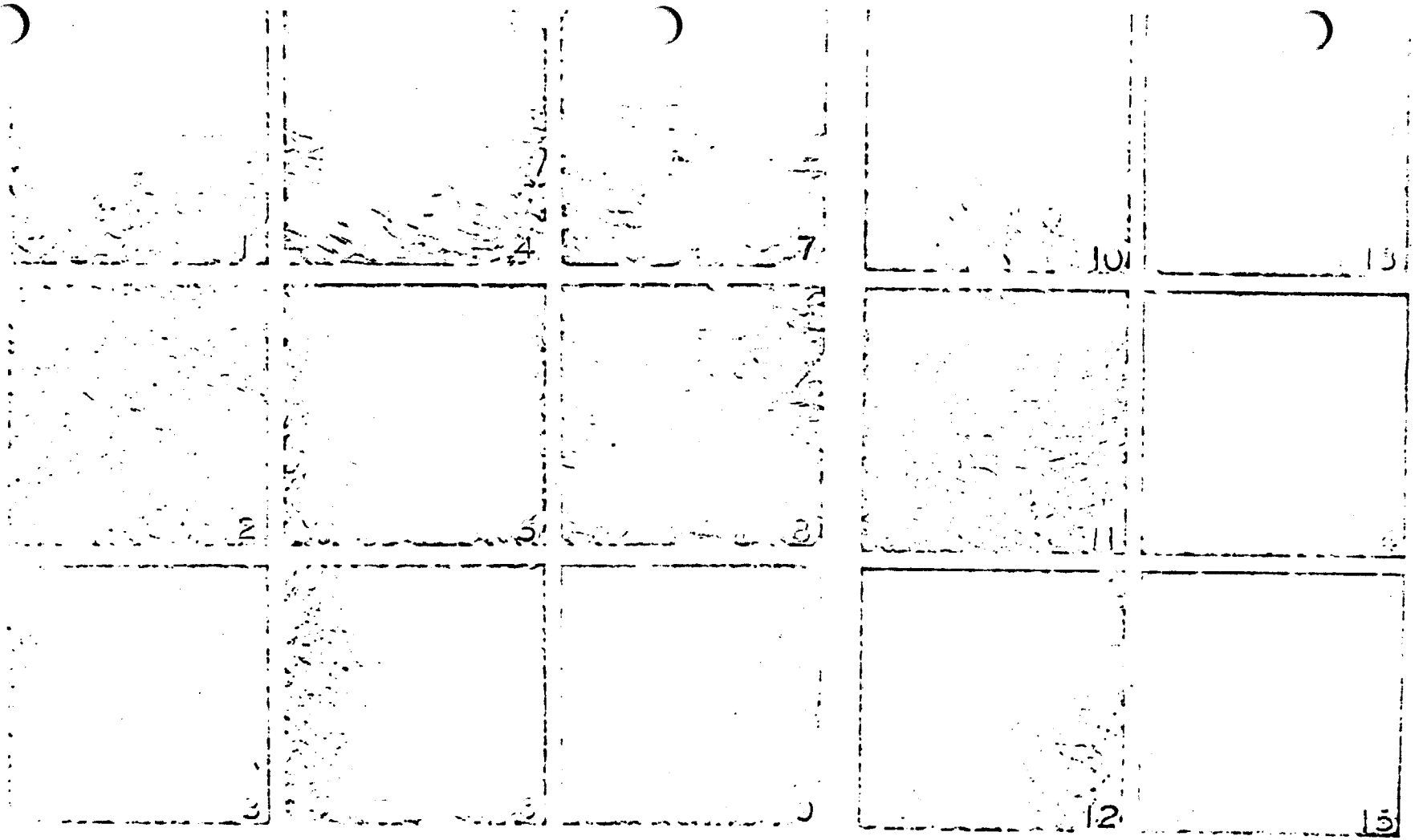

PLATE 1

EXPLANATION OF FIGURES

1, 4, 7, 10 and 13 are from the duodenum.
2, 5, 8, 11 and 14 are from the jejunum.
3, 6, 9, 12 and 15 are from the ileum.

Figures 1, 2 and 3 are fat diet mucosa; figures 4, 5 and 6 are carbohydrate diet mucosa; figures 7, 8 and 9 are liver supplemented diet mucosa; figures 10, 11 and 12 are [illegible] agar diet mucosa, and figures 13, 14 and 15 are balanced concentrated [illegible] diet.

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INHIBITION OF POLYNUCLEOTIDE KINASE BY AGAR, DEXTRAN SULFATE AND OTHER POLYSACCHARIDE SULFATES

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SUMMARY

In certain preparations of lambda DNA, the phosphorylation of their 5'-ends as catalyzed by polynucleotide kinase was inhibited. The inhibitor appeared to be due to traces of agar left in the DNA preparation. Of the several polysaccharides and their sulfuric acid esters tested for their inhibitory activity, all the polysaccharide sulfates are found to be strong inhibitors of the polynucleotide kinase whereas the non-sulfated polysaccharides are non-inhibitory. Since agar is sometimes used in growing the phages and dextran sulfate is often used in purifying the phages or DNA preparations, these potential inhibitors must be carefully removed from the final DNA preparations before a polynucleotide kinase reaction can be successfully carried out. However, the effect of small amounts of these inhibitors on the kinase can be counteracted by the addition of either polylysine or spermine.

Polynucleotide kinase is an enzyme which catalyzes the transfer of orthophosphate from ATP to the 5'-hydroxyl termini of polynucleotides. This enzyme was independently discovered by Richardson (1) and by Novagrodsky and Hurwitz (2,3) in T4 phage and T2 phage infected Escherichia coli B, and it has been widely used in studying the 5'-terminal structure of various nucleic acids as well as in making useful substrates for monitoring other reactions.

Inorganic phosphate (1,3) and pyrophosphate (3) have been reported to be inhibitors of polynucleotide kinase. In this communication, polysaccharide sulfates were found to be approximately 10,000 times more potent than inorganic phosphate or sulfate as inhibitors of the kinase, on a weight basis.

PREPARATIONS

The strains of lambda phage employed were lambda ind$^-$cI857 (4) and lambda c71 (5). The phages were grown and purified, and their DNA extracted and purified as described previously (4,6,7).

Polynucleotide kinase was purified according to the procedure of Richardson (1) with slight modification (7). The enzyme used for the experiments reported here had a specific activity of 20,000 to 30,000 units per mg.

RESULTS

Phosphorylation of the 5'-termini of lambda DNA - Purified lambda DNA was treated with alkaline phosphatase to remove the 5'-phosphate (7) and was used as substrate in a polynucleotide kinase catalyzed reaction. Results in Table I show that whereas the lambda DNA preparation 1 (DNA-1) accepted the theoretical amounts of ^{32}P, DNA-2 accepted none. Assay of the biological activity of the DNA by an infectivity assay (not given here) showed that both DNA preparations gave a comparable number of plaques per A_{260} of DNA indicating that the 5'-terminated cohesive ends are present in both preparations (6,7,8). The presence of an inhibitor of polynucleotide kinase in DNA-2 was suspected and this was confirmed by the observation that the phosphorylation of DNA-1 was strongly inhibited after the addition of small amounts of DNA-2 (Expt. 2). DNA-1 differs from DNA-2 in two aspects. First, the genetic background of the phages from which these DNA preparations were extracted were different. Second, the method of growing the phages was different: DNA-1 was extracted from phage lambda cI857 grown in liquid medium and DNA-2 was extracted from phage lambda c71 grown in agar containing solid medium. Since the main difference in the composition of the two growth media was agar, it was suspected as the inhibitor. Indeed, addition of small amounts of agar to the polynucleotide kinase reaction mixture resulted in strong inhibition of the phosphorylation of both DNA-1 and a partial digest of salmon sperm DNA (Expt. 3 and 4). It may be estimated that approximately 1500 μg of DNA-2 was obtained from a phage preparation starting with 500 ml of soft agar containing medium, it may be calculated that approximately 0.003% of the original agar was present in the final DNA-2 preparation.

Effect of polysaccharides and polysaccharide sulfates on polynucleotide kinase - Agar is a sulfuric acid ester of a complex polysaccharide, composed

TABLE I

Phosphorylation of the 5'-Termini of DNA and Inhibition of Polynucleotide Kinase by Agar

The reaction mixture (0.3 ml) contained 70 mM Tris buffer, pH 7.6, 12 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.03 mM [γ-^{32}P] ATP (1 x 10^9 cpm per μmole), and DNA as indicated. Ten units of polynucleotide kinase were used for Experiments 1, 2, 3, and 0.1 unit for Experiment 4. After incubation at 37° for 60 min. the labeled DNA was precipitated with perchloric acid, washed, filtered and counted (7).

Expt.	DNA preparation*	μg DNA per tube	μg agar added	Moles of ^{32}P incorp/ 10^5 moles DNA-P**	% inhibition
1	DNA-1	50		2.2	0
	DNA-2	50		0	100
2	DNA-1 + DNA-2	50 + 5		1.0	55
	DNA-1 + DNA-2	50 + 15		0.4	82
3	DNA-1	50	-	2.1	0
	"	50	0.3	0.9	57
	"	50	1.0	0.3	86
4	Salmon DNA digest	10	-	1200	0
	"	10	0.3	370	69
	"	10	1.0	60	95

* Lambda DNA preparation 1 (DNA-1) was purified from lambda cI857 phage and lambda DNA preparation 2 (DNA-2) was purified from lambda c71 phage. Both preparations had been treated with alkaline phosphatase to remove the 5'-phosphate. The salmon DNA digest used in Experiment 4 was prepared by digesting native salmon sperm DNA with micrococcal nuclease until 30% of the DNA was converted to acid-soluble products (1). The DNA digest was extensively dialyzed against 1 M KCl and then against 0.02 M KCl before use.

** The molecular weight lambda DNA was taken as 3.3 x 10^7, therefore, 1 mole of lambda DNA contains 10^5 moles of DNA-P (or 10^5 moles of mononucleotides). From the phosphorylation data, the average molecular weight of DNA in the salmon DNA digest was calculated to be 6 x 10^4. The values for incorporation have been corrected for blank values obtained in the absence of the kinase (7).

mainly of galactose units. Since commercial agar is not highly purified, it was of interest to determine whether polysaccharide sulfate was responsible

for the inhibition of the polynucleotide kinase and if so, whether the polysaccharide portion or the sulfate group was primarily responsible. Agar can be separated into agaropectin and agarose components and the latter contains almost no sulfate groups (10,11). Agarose (with less than 0.1% S) is much less inhibitory than agar (with 0.84% S, from Difco Company) suggesting that it is the sulfate group of agar which is responsible for the inhibition of polynucleotide kinase (Table II). This interpretation is confirmed by comparing the effect of highly purified dextran sulfate and dextran on the kinase. Dextran sulfate contains approximately twenty times as much sulfur and possesses approximately twenty times stronger inhibitory activity on the kinase as compared to agar, whereas dextran contains no sulfur and is non-inhibitory even at much higher concentrations. Heparin has a high sulfur content and it is a strong inhibitor of the kinase. In fact, the extent of inhibition appears to be approximately proportional to the sulfur content of these polysaccharide sulfates, independent of their molecular weights (2×10^4 for heparin and 3.6×10^7 for amylopectin sulfate).

Since these polysaccharide sulfates are all polyanions at pH 7.6, the pH of the kinase reaction mixture, other polyanions were tested for their action on the kinase. Hyaluronic acid and polygalacturonic acid at much higher concentrations showed little or no inhibition on the kinase activity. Lipopolysaccharide isolated from Salmonella typhimurium (gift of Drs. Falaschi and Kornberg, see reference 14) was not inhibitory at a concentration of 10 μg per ml.

Comparison of the inhibitory action of dextran sulfate with inorganic sulfate or phosphate - It was concluded from the data just presented that it is the sulfate group on the polysaccharide rather than the polysaccharide itself which is responsible for the inhibition of polynucleotide kinase. Inorganic sulfate was then tested as a possible inhibitor. Although Na_2SO_4 is an inhibitor of polynucleotide kinase it is much less inhibitory than dextran sulfate. At least 10,000 times higher level (on weight basis) of Na_2SO_4

TABLE II

Effect of Polysaccharides and Polysaccharide Sulfates on Polynucleotide Kinase

The incubation conditions were the same as in Table I, except that the specific activity of labeled ATP was lowered to 1×10^8 cpm per μmole. In each tube 8 μg of the salmon DNA digest and 0.1 unit of polynucleotide kinase were used. Incubation was at 37° for 30 min.

Additions	% Sulfur	μg per ml	Counts/min incorporated	% inhibition
None	-	-	10,100	0
Agar	0.84	1	5,100	50
		3	1,800	82
Agarose	<0.1	3	9,600	4
		20	8,300	17
Dextran sulfate*	17	0.06	2,000	80
		0.3	240	99
Dextran*	0	20	10,000	0
Amelopectin sulfate*	14.7	0.06	2,800	72
		0.3	500	95
Amelopectin*	0	3	9,900	0
Heparin**	13-15	0.06	3,200	68
		0.3	1,300	87
Hyaluronic acid*	0	3	9,000	10
Polygalacturonic acid**	0	3	9,500	5

* Gifts of Dr. P.S. Cammarata (12). Dextran sulfate and dextran were also purchased from Pharmacia Co. The average molecular weight of amylopectin sulfate is 3.6×10^7, that of the two samples of dextran sulfate is 2×10^6 and 5×10^5. The % inhibition by 0.02 μg of dextran sulfate is the same with both samples.

** Heparin and polygalacturonic acid was purchased from Sigma Co., and Mann Research Laboratories, respectively.

(K_i = 600 μg/ml) than dextran sulfate (K_i = 0.05 μg/ml) was needed to produce the same inhibition. Therefore, the attachment of the sulfate group to the

TABLE III

Reversal of Agar or Dextran Sulfate Inhibition of Polynucleotide Kinase by Poly-L-Lysine or Spermine

The incubation conditions were the same as in Table II. The order of addition is as follows: Buffer, cofactors and salmon DNA digest in a mixture; compound listed under First Addition; compound listed under Second addition; polynucleotide kinase.

First Addition	μg per ml	Second Addition*	μg per ml	Counts/min incorporated	% inhibition
None	-			8,000	0
Dextran sulfate	0.15			1,100	86
"	0.15	Polylysine	0.15	2,600	67
"	0.15	"	0.30	7,800	3
"	0.15	Spermine	0.90	4,600	43
"	0.15	"	1.50	7,400	7
None	-			8,400	0
Agar	3.0			900	90
"	3.0	Poly-L-lysine	1.0	6,200	26
"	3.0	"	3.0	7,600	9

* Poly-L-lysine and spermine were purchased from Sigma Co. After the addition of these compounds, the reaction mixture was preincubated at 37° for 15 min. to allow them to react with the inhibitor before polynucleotide kinase was added. The polyamines added separately to the enzyme assay did not affect the polynucleotide kinase activity.

dextran or other polysaccharide molecules resulted in a much more potent inhibitor of the kinase. It is likely that the dextran portion of the dextran sulfate participates in increasing the affinity between the sulfate groups

and the enzyme molecule. A second difference between dextran sulfate and Na_2SO_4 is that the former is a non-competitive inhibitor with respect to both DNA and ATP whereas the latter is non-competitive with respect to DNA but is a competitive inhibitor with respect to ATP.

The K_i for inorganic phosphate as an inhibitor of the kinase as well as the mode of inhibition appears to be similar to that of inorganic sulfate ($K_i = 4 \times 10^{-3}$ M).

DISCUSSION

Polysaccharide sulfates are found to be potent inhibitors of polynucleotide kinase. Since microgram levels can give strong inhibition of the polynucleotide kinase, it is essential that the DNA preparations be sufficiently free of the polysaccharide sulfates. If the phage was concentrated with the dextran sulfate-polyethylene glycol two-phase system (13), or if the DNA was purified with dextran sulfate-methylcellulose system (13), the dextran sulfate must be removed. However, the effect of small amounts of these inhibitors on polynucleotide kinase can be counteracted by the addition of either polylysine or spermine (Table III). Since these inhibitors are polyanions at neutral pH, it is not surprising that their inhibitory effect can be reversed by the addition of cationic compounds such as polylysine and spermine. A similar neutralization effect of polyamines on a lipopolysaccharide inhibitor of a DNA methyl transferase has been reported (14).

ACKNOWLEDGEMENT

The work reported in this paper was supported by grant GB-13912 from the National Science Foundation and grant CA-08999-05 from the National Cancer Institute of the National Institutes of Health.

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