BENTONITE AND CLAY #75

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O N

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INFORMATICS INC.
6000 Executive Boulevard
Rockville, Maryland 20852

BENTONITE AND CLAY

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BENTONITE AND CLAY

Summary

Kaolin, an anticaking agent, and bentonite, a bonding agent for pelleted feeds, have been known to the food industry for some time. The word kaolin is "a transliteration of two Chinese characters which mean high mountain range". When the Chinese pronounced these characters, it sounded to foreigners like "kao-ling or gao-ling" (311). Bentonite was so named because of its discovery in the Fort Benton formation of the Upper Cretaceous in Wyoming (528). Both materials are aluminum silicates, kaolin being hydrated.

No studies were found which specifically investigated the absorption or metabolism of either bentonite or clay. Gleason et al. (489) listed kaolin as an inert substance in the Clinical Toxicology of Commercial Products.

Kaolin has been given in several hundred gram doses as an adsorbent for intestinal disorders (489); Damrau (38) found bentonite to be useful in the same capacity. As early as 1923, Braafladt (311) reported that ingested kaolin changed the intestinal flora of rats, dogs, and man from a predominantly proteolytic type to an aciduric type.

In a study by Olesen (524), bentonite lowered the pepsin-activity and exerted a protective effect against gastric ulcers in rats.

Both leucine amino-peptidase and various oxidative enzymes in the intestinal mucosa increased their activity after rats were fed 66 and 80% kaolin for 12 weeks; Riecken et al. (418) associated these changes with an increased intestinal absorptive capacity.

Hitchens (360) reported that kaolin given i.p., s.c., or p.o. elevated the pain threshold of rats subjected to the rat pressure pain test. Kaolin has been used by Coulon et al. (319) as an inflammatory agent.

Inhalation of kaolin dust has been known to cause bronchospasm and disproteinemia (120).

Laughland and Phillips (137) reported that dietary sodium bentonite at levels of 0.5, 1.0, 1.5, or 2.5% impaired liver storage of vitamin A. An in vitro study by these authors (136) indicated that vitamin A alcohol was dehydrated in the presence of bentonite as well as physically bound to the bentonite.

Brune and Eger (314) stated that kaolin in the diet improved vitamin D_3 resorption in D_3-hypervitaminotic rats.

Acute toxicity, short term feeding, and special studies were performed on chicks, mice, rats, monkeys, and man. No long term studies could be located. A table of acute toxicity data is presented on page 9.

Tripsa (441) found that intratracheal instillation of up to 40 mg kaolin produced alveolitus in rats within 3-11 months. Intravenous injection of 40 mg of kaolin resulted in lesions in the liver and spleen; i.p. injection produced nodules in the omentum, mesentery, and intestinal loop.

Introduction of 70-100 mg Askansk clay dust into the trachea of rats caused moderate, diffuse pneumosclerosis. The development was followed by Kipiani (120) for 9 months.

Several short-term studies involving chicks are reviewed. Briggs and Fox (21) fed day-old New Hampshire chicks a glucose-casein diet plus 0 or 5% kaolin for 4 weeks without ill effect. However, chicks fed this diet plus 0, 1, 2, 3, 5, or 10% bentonite showed vitamin A deficiency symptoms at the 3% level or higher. Additional vitamin A overcame the deleterious effects. The coarseness of the bentonite affected the toxicity; no untoward effects were found at a sieve size of 90 mesh or coarser unless the bentonite constituted 20% of the diet.

A study by Day et al. (40) showed that calcium bentonite restricted growth and feed efficiency when fed to chicks at a 10% level for 4 weeks. Five and ten percent kaolin and 5% calcium bentonite did not reduce growth. In a second study, growth was depressed by both clay and bentonite at the 5% level. The authors also found a level of 1-2% of kaolin or bentonite improved growth slightly.

The feeding of a basal diet plus 0, 6, 12, 18, 24, 30, 36 and 42% kaolin to chicks produced a decreased gain in weight at the 18% level and above. Sibbald et al. (427) found the feed consumption to be high for all animals.

Wilson (290) fed groups of 4 weanling mice a semisynthetic diet plus 0, 10, 25, or 50% bentonite for 61 days. The growth rate was reduced for all bentonite groups, especially at the 50% level. The livers were fatty, too. Further study indicated that the growth retardation was not due solely to bulk or partial inanition. The results appeared to indicate a nutritional deficiency that was partly relieved by additional dietary choline chloride; the cation-exchange properties of bentonite were held responsible.

Vasileva (273) fed 0 or 30% natural Uzbek bentonite in either protein-sufficient or protein-deficient diets to male mice for 7 or more days. Bentonite-fed mice grew less or lost up to 25% of their original weight and developed fatty livers; these effects were more pronounced in the protein-deficient mice. Because the administration of scrapings from a pig's intestine had a lipotropic action, the author felt that bentonite absorbed some lipotropic agent(s) in the intestine.

Although growth was unaffected, Laughland and Phillips (137) found that liver weight was significantly reduced in rats fed 0.5, 1.0, 1.5, or 2.5% sodium bentonite in a fox cube diet for two weeks. Groups of 20 hooded rats were fed by Dowling et al. (330) a standard diet which included 0, 66, or 80% powdered kaolin for 9-12 months. Growth was de-

pressed while feed consumption increased by a factor of 3 or 4; cecum and colon weights increased.

According to Kennedy (500), weight loss of rats on a 50 or 75% kaolin diet depended on their original weight. Animals weighing less than 300 g on a 50% diet and 200 g on a 75% diet could maintain their weight over a 14-day period.

No ill effects were noted by Weinstein and Weiss (447) after feeding 2 or 4 g kaolin to rats for 4 weeks.

Some pregnant women from rural parts of the southeastern United States experienced a craving for clay and ate from 6 to 130 g daily. Several of the women believed the clay prevented the nausea and vomiting associated with pregnancy (335).

Wilson (291) found that hepatomas developed in 11 out of 12 mice fed a 50% 50% bentonite-basal diet for up to 480 days. Because the liver damage resembled that of animals fed a choline-deficient diet, it was suggested that bentonite acts as an exchange silicate in the intestine, removing choline and other nutrients. In a second paper Wilson (292) went into the histological aspects in greater detail.

Leu and Ruttner (382) gave groups of 30 mice an intraperitoneal injection of 0.5 ml of a 1% suspension of kaolin; dust cell granulomas were formed.

Granulomatous lesions found in the prepyloric region of a man who had ingested a kaolin preparation over a long period of time induced Cohn et al. (318) to test this preparation in rabbits. A small area of the stomach was curetted and the animals fed a ration containing an unspecified amount of kaolin. No gross mucosal lesions were noted, but multinucleated giant cells were found.

Accidental penetration of the pharynx or cheek pouch of sixteen macaques while dosing with an anti-diarrheal kaolin suspension produced benign granulomas (417).

Potts (530) described two subcutaneous granulomas resulting from the application of kaolin poultices to the breast and coccygeal region of a young woman.

BENTONITE AND CLAY

Chemical Information

I. Nomenclature

A. Common Names

Bentonite

Wilkinite; soap clay; mineral soap

Clay (Kaolin)

Bolus alba; China clay; white bole; argilla; terra alba; porcelain clay

B. Chemical Names

Bentonite

No chemical names for bentonite were encountered in the literature searched.

Clay (Kaolin)

No chemical names for clay were encountered in the literature searched.

C. Trade Names

Bentonite

Black Hills Western Bentonite
Dixie Bond Southern Bentonite
Bentonite Magma
Magbond
Panther Creek Bentonite
Southern Bentonite
Tixoton
Volclay

Clay (Kaolin)

No trade names for clay were encountered in the literature searched.

D. Chemical Abstracts Services Unique Registry Number

Bentonite

001302-78-9

Clay

001302-87-0

Kaolin

001302-58-7

II. Empirical Formula

Bentonite

$Al_2O_3 \cdot 4SiO_2 \cdot H_2O$

Clay (Kaolin)

$H_2Al_2 \cdot Si_2O_8 \cdot H_2O$

III. Structural Formula

Bentonite

No structural formula for bentonite was encountered in the literature searched.

Clay (Kaolin)

No structural formula for clay was encountered in the literature searched.

IV. Molecular Weight

Bentonite

No molecular weight for bentonite was encountered in the literature searched.

Clay (Kaolin)

No molecular weight for clay was encountered in the literature searched.

V. Specifications

A. Chemical

Bentonite

Montmorillonite is the predominant constitutent in bentonite, but other clay minerals such as illite and

kaolinite may be present, as well as a variable amount of nonargillaceous detrital minerals. Although certain bentonites appear relatively pure, the content of nonargillaceous minerals is very rarely below ten percent. Moreover, cristobalite is frequently present in bentonites. The composition of montmorillonite itself varies from one bentonite to another, either in its lattice or in the nature of the exchangeable ions.

Clay (Kaolin)

Analyses of Typical Kaolins, %

Type of kaolin	SiO_2	Al_2O_3	Fe_2O_3	FeO	CaO	MgO	Alkalies	TiO_2	H_2O
sedimentary (Fla.)	47.0	36.8	0.8		0.2	0.2	0.2	0.2	15.0
crude residual (N.C.)	62.4	26.5	1.4		0.6		1.0		8.8
washed residual (N.C.)	45.8	36.5	0.3	1.1	0.5		0.3		13.4
English china clay	48.3	37.6	0.5		0.1		1.6		12.0
sedimentary (Fla.)	45.0	38.7	0.4				0.5	1.4	13.6
sedimentary (Ga.)	45.0	38.1	0.6					1.7	14.7

B. Food Grade

Bentonite

No food grade for bentonite was encountered in the literature searched.

Clay (Kaolin)

Loss on ignition	Not more than 15%
Limits of Impurities	
Acid-soluble substances	Not more than 2%
Arsenic (as As)	Not more than 3 ppm (0.003%)
Carbonate	Passes test
Heavy metals (as Pb)	Not more than 40 ppm (0.004%)
Iron	Passes test
Lead	Not more than 10 ppm (0.001%)
Sulfide	Passes test

C. Official Compendia

Food Chemicals Codex

Kirk - Othmer Encyclopedia of Chemical Technology

VI. Description

A. General Characteristics

Bentonite

A very fine, pale buff or cream-colored powder, odorless, and with a slightly earthy taste. The color in the massive condition varies from yellowish-white to almost black. The bentonites are of variable color, ranging from white to gray, yellow, green, and blue. They have a waxy luster when fresh.

Clay (Kaolin)

A purified clay consisting mainly of alumina, silica, and water. It occurs as a fine, white to yellowish white or grayish powder having an earthy taste. It becomes darker and has a distinct clay-like odor when moistened.

B. Physical Properties

Bentonite

Bentonite is insoluble in water but when mixed with 8 to 14 parts of the latter it swells to produce a slippery paste resembling petroleum jelly. The consistency of the gel may be regulated by varying the amount of water. Suspensions containing smaller amounts of bentonite are quite permanent, particularly if a fine-particle variety of the clay is used. In aqueous suspensions, the individual particles of bentonite are negatively charged. The pH of the medium in which bentonite is dispersed has a marked effect on the stability of the suspension; above about pH 7 suspensions are considerably more stable than below this pH.

Clay (Kaolin)

Kaolin is insoluble in water, cold acids or in alkali hydroxides.

C. Stability

Bentonite

Store in well-closed containers.

Clay (Kaolin)

Store in well-closed containers.

VII. Analytical Methods

Sadik et al. described a x-ray diffraction method for the identification of kaolin and bentonite. The unit cell structure of kaolin has two layers,

stacked one over the other, with the flat sides together. One layer is composed of Al_2O_3 molecules and the other of SiO_2 molecules. In bentonite, the unit cell structure consists of one layer of Al_2O_3 sandwiched between 2 layers of SiO_2. These differences permit differentiation of the materials by x-ray diffraction; x-ray diffractograms are included (422).

Angino presented the far infra-red spectra of montmorillonite, kaolinite, and bentonite; this method is applicable only to qualitative analysis (3).

VIII. Occurrence

Natural inorganic sources

Bentonite

Large deposits of bentonite have been found in the U.S., Canada, Mexico, China, Japan, France, Great Britain, Germany, Italy, Greece, the U.S.S.R., Poland, Hungary, North Africa, South Africa, and New Zealand.

Clay (Kaolin)

The best clays, the China clays, are found in England, the United States, France, Czechoslovakia, Germany, and Japan. The china clay of commerce is obtained by screening and elutriation of the crude mined material, the larger particles of quartz, mica and partially altered feldspar being thus removed.

Biological Data

I. Acute Toxicity

Substance	Animal	Sex & No.	Route	Dosage mg/kg Body Weight	Measurement	Ref.
Bentonite	(No information available)					
Kaolin	Rat	10 male	i.p.	170 mg/kg	LD_{50}	360
Kaolin	Rat	10 male	s.c.	1600 mg/kg	LD_{50}	360
Kaolin	Rat	10 male	per os	800 mg/kg	LD_{50}	360
Kaolin	Rat	12-16	per os	148.5 g/kg	ED_{50}	310
Kaolin	Rat	12-16	per os	110 g/kg	ED_1	310
Kaolin	Rat	12-16	per os	188 g/kg	ED_{100}	310

ED_{50} is the dose which killed 50% of the rats by bowel obstruction

ED_1 is the dose which killed 1% of the rats by bowel obstruction

ED_{100} is the dose which killed 100% of the rats by bowel obstruction

A. Bentonite

No Information Available

B. Kaolin

Rats

Death from bowel obstruction due to intragastrically administered kaolin was studied in male CBL-Wistar rats weighing 90-100 g. Groups of 12-16 animals were given doses of 100, 110, 120, 140, 145, 150, 155, 160, 175, and 210 g/kg of a 100% (w/v) suspension; 14 control rats received 150 ml/kg of distilled water. A maximum of 120 ml/kg was given at a time; greater quantities produced gastric perforation. Of 140 rats treated, 47 were found to have expired from aspiration or gastric rupture. Animals were observed for 1 month. Body weight, food and water consumption, temperature, and urinary volume, glucose and protein output, and pH were measured. Autopsy was performed on the following organs: adrenal glands, brain, cardiac and pyloric stomachs, small bowel, cecum, colon, heart, kidneys, liver, lungs, muscle, skin, spleen, testes, thymus gland, and the residual carcass (310). The dose which killed 50% of the rats by bowel obstruction, the ED_{50}, was found to be 148.5 ± 16.8 g/kg. An ED_1, of 110 ± 17 g/kg and an ED_{100} of 188 ± 17 g/kg were also determined. Gagging and retching were observed, the animals became tense, and there was a series of abdominal muscle fasciculations beginning at the pubis and passing craniad with some extension to other skeletal muscle. The animals were also listless, withdrawn,

failed to respond to stimuli, made compulsive movements and had pallor of the ears and tail. Their mouths were dry, bloody, and tense, with a brownish incrustation about the nose and mouth; diarrhea developed. Food and water intake, body weight gain, and chronic temperature were markedly depressed. Urinary volume obtained over a 24-hour period was markedly decreased and there was a marked glucosuria and slight aciduria. None of the clinical signs were dose dependent (310).

Bowel obstruction was the predominant feature observed during autopsy; kaolin impacted in the stomach produced necrosis particularly of the stratified squamous epithelium and muscularis externa along with marked inflammation. The spleen, kidneys, and lungs were hypovascular; the adrenals were hypertrophied. The cause of death was respiratory failure following circulatory stasis induced by bowel obstruction (310).

Tripsa gave 4 groups of 14-19 rats (weighing 160-190 g) 10 mg kaolin +30 mg SiO_2, 20 mg kaolin + 20 mg SiO_2, 30 mg kaolin + 10 mg SiO_2, or 40 mg kaolin by intratracheal instillation. Control animals received 10, 20, and 40 mg of SiO_2 only. Groups of animals were sacrificed at 4, 7, 9, and 11 months. During the first 3-5 months the 10, 20, and 30 mg kaolin groups were histologically similar to the group receiving 40 mg of pure SiO_2 and were characterized by alveolitis of moderate severity. Cellular nodules appeared between 5-7 months. All the kaolin mixtures induced a more advanced silicosis than did the corresponding amount of SiO_2 present in the mixture when given alone; fibrosis appeared earlier, too. Kaolin alone produced similar results (441).

Rats were also injected i.v. with 40 mg kaolin in doses no larger than 6.8 mg/injection. Lesions of the liver and spleen evolved over a 12-18 month period. Intraperitoneal injection of 50 mg kaolin resulted in nodules in the omentum, mesentery, and the intestinal loop (441).

Hitchens determined the acute toxicity of kaolin in male albino Sprague-Dawley rats (60-80 g). Ten rats were used per dose (not specified further), and the LD_{50}'s were determined after 7 days. The volume administered was 0.1 ml kaolin in a 0.5% gum tragacanth suspension per 10 g of body weight for intraperitoneal, subcutaneous, and oral routes. The LD_{50}'s are presented below (360).

	Intraperitoneal	Subcutaneous	Oral
LD_{50} % concent.	1.7%	16%	8%
LD_{50} mg/kg	170 mg/kg	1500 mg/kg	800 mg/kg

Introduction of 70-100 mg of Askansk clay dust into the trachea of white rats lead to a moderate, diffuse pneumosclerosis, with formation of dust cell foci. Development was followed over a 9-month period (120).

II. Short Term Studies

A. Bentonite

Chicks

Briggs and Fox reported that the feeding of bentonite to chicks for 4 weeks resulted in vitamin A deficiency. Day-old New Hampshire chicks were provided with a diet containing 61.5% glucose, 20% vitamin-free casein, adequate vitamins, plus 0 or 5% bentonite. The following table shows a decreased weight gain at the 5% level (21).

TABLE 1.—*Effect of bentonite on mortality and weight of chicks fed synthetic diets*
(Summary of 10 experiments with mixed sexes)

Supplement to diet C2	Number started	Number of survivors at 4 weeks	Gm. weight of survivors at 4 weeks
None	60	60	347
5% bentonite	60	15	165

Gross signs of vitamin A deficiency appeared within 2-3 weeks. The second table demonstrates the activity of additional vitamin A in overcoming the harmful effects of bentonite (21).

—*Effect of various supplements on bentonite toxicity*
(6 chicks started per group)

Group	Supplement to diet C2	No. survivors at 4 weeks	Gm. wt. of survivors at 4 weeks and standard error*	Feed efficiency†
	Series 63—females			
1	None	6	348±12	0.590
2	Minus vitamin A	1	181	.474
3	5% bentonite	1	190	.484
4	5% bentonite+5 mg. β-carotene/kg.	6	300±18	.532
5	5% bentonite+10×all B vitamins except choline	1	190	.481
	Series 67—females			
6	None	6	380±26	.592
7	5% bentonite	2	182±12	.364
8	5% bentonite+27 mg. vitamin A acetate/kg.	6	349±24	.570
9	5% bentonite+vitamin A acetate injected 3× weekly subcutaneously (equivalent to that consumed by preceding group)‡	6	370±13	.596
10	5% bentonite+45 mg. β-carotene/kg.	5	362±34	.576
11	5% bentonite+3 mg. "stabilized" vitamin A/kg.§	6	348±12	.565
12	5% bentonite+90 mg. vitamin E/kg.	0	(all dead)	—
13	5% bentonite minus corn oil	6	338±30	.533
14	5% bentonite minus corn oil+3% Crisco	3	230±19	.433

* Standard error calculated by method of Mantel (1951).

† Feed efficiency = $\frac{\text{total weight gained (of all animals)}}{\text{total feed consumed}}$.

‡ Vitamin A acetate in 50 percent alcoholic solution for the first 2 weeks and in corn oil from 2 to 4 weeks.

§ Supplied in the form of NOP-DRY, Nopco Chemical Co., Harrison, N. J.

A third study revealed that the level of vitamin A deficiency was dose related, as shown below.

TABLE 3.—*Effect of various levels of bentonite*
(6 chicks per group)

Level of bentonite added to diet C2	Gm. wt. at 4 weeks, standard error, and survivors*		
	Series 60 (females)	Series 69 (females)	Series 71 (males)
0	354 ± 8 (6)	361 ± 20 (6)	376 ± 22 (6)
1%	324 ± 22 (6)	347 ± 6 (6)	365 ± 21 (6)
2%	—	—	317 ± 26 (6)
3%	—	205 ± 10 (3)	194 ± 14 (2)
5%	133 ± 21 (2)	115 ± 4 (2)	—
10%	—	(all dead)	—

* Number of survivors indicated in parenthesis.

The 2% level induced the characteristic eye symptoms of vitamin A deficiency. At the 3-10% level, pronounced deficiency symptoms (urates in the ureters, poor growth, eye problems, and high mortality) were observed.

The following table illustrates the relation of bentonite particle size to toxicity (21).

TABLE 4.—*Effect of particle size of bentonite and other adsorbing agents on growth and mortality of chicks*
(6 chicks started per group)

Supplement to diet C2	Sieve number*	Sample†	Number of survivors at 4 weeks	Gm. wt. of survivors at 4 weeks and standard error	Vitamin A deficiency signs
None	—	—	6	344 ± 19	—
5% Na bentonite	200	A	1	135	+
5% Na bentonite	200	B	1	250	+
5% Ca bentonite	200	C	1	133	+
5% Na bentonite	200	D	3	168 ± 20	+
5% Na bentonite	90	E	6	332 ± 25	−
20% Na bentonite	90	E	0	(all dead)	+
5% Na bentonite	80	F	6	326 ± 22	−
5% Na bentonite	50	G	6	384 ± 16	−
5% kaolin	200	H	5	346 ± 24	−
5% Norite	200	I	0	(all dead)	?

* The sieve number gives the number of openings per linear inch.
† Sample A was used in all previous experiments. The other samples were commercial bentonite. Samples D, E, and G were the same bentonite but of different grind.

When the bentonite was 90 mesh or coarser, no untoward effects were noted unless extremely high levels were fed (20%). Chemical analysis of the synthetic diet indicated that there was appreciable disappearance of vitamin A in the presence of bentonite (21).

Mice

Wilson fed groups of 2 male and 2 female weanling mice (strains BUA, BUB, and BUC) a basal semisynthetic diet plus 0, 10, 25, or 50% bentonite for 61 days. Those on the 10 and 25% bentonite grew slightly slower than controls, but the 50% group had a greatly retarded growth rate. The livers of these last animals contained abundant fat vacuoles. When either 50% Ruffex (cellulose) or 50% barium sulfate was given, growth was nearly normal. However, when 50% montmorillonite (a clay forming the principal constituent of bentonite and fuller's earth) was fed, growth was extremely retarded. This indicated to Wilson that the bentonite effect was not due solely to bulk (290).

Further study revealed that partial inanition was not responsible for the growth depression. Groups of 2 young adult mice (70-day-old) were fed the basal diet plus 0, 50% Ruffex, or 50% bentonite for 16 days. Feed intake and growth were measured. The two experimental groups had somewhat decreased feed consumptions, but growth on Ruffex was only slightly depressed, whereas the bentonite group suffered severe growth reduction (290).

In another series 50% bentonite replaced 35% cornstarch and 15% Crisco instead of a 50-50 bentonite-basal mixture. Because growth was only moderately retarded and the livers normal in the first group, Wilson believed the bentonite effect to be due to a nutritional deficiency. When 0.15% choline chloride was added to the basal diet, moderate though subnormal growth was noted; livers were normal. However, methionine, a lipotropic agent, did not relieve these symptoms. Wilson concluded that bentonite, a cation-exchange silicate, removes choline from the intestinal contents producing a deficiency, but other materials may also be involved (290).

Natural Uzbek bentonite comprised 0 or 30% of a synthetic diet fed to male mice, weighing 18-21 g, of the C57BL, C3N, and C3NA strains. Both protein-sufficient and protein-deficient diets were given for at least 7 days. The bentonite mice either grew less than controls or lost up to 25% of their original weight in 7 days; also there was an increase in the fat content of the liver. Studies indicated that a 7 day period was required for the development of fatty liver degeneration. Effects were more pronounced in those mice fed the protein-deficient diet. A definite lipotropic effect was noted following the administration of scrapings from a pig's small intestine in these mice. Vasil'eva stated that bentonite absorbs unspecified lipotropic agents in the intestine (273).

Rats

Laughland and Phillips fed groups of 10 male and 10 female rats a commercial fox cube diet plus 0, 0.5, 1.0, 1.5, or 2.5% sodium bentonite. After two weeks, the livers were removed and analyzed. Body weight was not affected during this period, but the liver weight (total liver weight or grams liver weight per 100 g body weight) was significantly reduced in animals fed bentonite (137).

B. Kaolin

Chicks

Day et al. tested the effect of feeding graded levels of kaolin and calcium bentonite to groups of 25 broiler chicks (day-old Vantress X Cobb) for 4 weeks. Diets and results are presented in the table below:

—The influence of different sources and levels of pellet binding agents on the performance of male chicks (Exp. 1)

Dietary treatment	Four week results		
	Mean wt., g.[1]	Feed/weight[1]	Prod. cal./kg. meat
Basal	636[ab]	1.58[a]	3,716
Basal+kaolin A, 5%	653[a]	1.66[ab]	3,709
Basal+kaolin B, 5%	624[ab]	1.68[ab]	3,753
Basal+Ca bentonite, 5%	615[b]	1.66[ab]	3,709
Basal+kaolin A, 10%	642[ab]	1.76[bc]	3,725
Basal+Ca bentonite, 10%	562[c]	1.84[c]	3,894

[1] Means within a column and without a common superscript are significantly different (P<.05).

Kaolin A and B are Mistrex 60 and 77; they were not specified further. Only calcium bentonite at 10% proved harmful; growth was restricted and the caloric efficiency decreased (40).

In a second study, replicate lots of 80 chicks were fed the diets shown below for 8 weeks.

—The influence of different pellet binding agents on the performance of broilers (Exp. 2)

Dietary treatment	Mean wt.[1], g.		Feed/weight[1]		Prod. cal./kg. meat	
	5 wk.	8 wk.	5 wk.	8 wk.	5 wk.	8 wk.
Basal	830[a]	1,524[a]	1.88[a]	2.26[a]	4,286	5,240
Basal+kaolin A, 5%	812[ab]	1,497[b]	1.98[b]	2.40[b]	4,288	5,289
Basal+kaolin B, 5%	816[a]	1,497[b]	1.96[b]	2.39[b]	4,244	5,267
Basal+Ca bentonite, 5%	771[b]	1,433[c]	2.04[c]	2.49[c]	4,418	5,487

[1] Means within a column and without a common superscript are significantly different (P<.05).

At 8 weeks, growth and feed efficiency were depressed by all three silicates, especially calcium bentonite. The basal diet used here contained less energy than the basal diet in the first experiment. Growth reduction is less apt to occur with a high energy diet; this may explain why the effects were more severe in the second experiment than in the first (40).

A third experiment is outlined on the following page.

—*The influence of source and levels of pellet binders on the performance of male chicks (Exp 3)*

Dietary treatment	Four week results		
	Mean wt., g.[1]	Feed/ weight[1]	Prod. cal./ kg. meat
Basal	632[a]	1.58[ab]	3,716
Basal+kaolin A, 1%	658[abc]	1.56[a]	3,630
Basal+kaolin A, 2%	681[c]	1.60[abc]	3,683
Basal+kaolin A, 3%	632[a]	1.63[bc]	3,718
Basal+kaolin B, 1%	672[bc]	1.58[ab]	3,678
Basal+kaolin B, 2%	642[ab]	1.60[abc]	3,683
Basal+kaolin B, 3%	636[a]	1.59[ab]	3,628
Basal+Ca bentonite, 1%	662[abc]	1.58[ab]	3,678
Basal+Ca bentonite, 2%	657[abc]	1.58[ab]	3,637
Basal+Ca bentonite, 3%	639[a]	1.65[c]	3,764

[1] Means within a column and without a common superscript are significantly different (P <.05).

The basal diet was the same as in the first experiment. The growth rate tended to improve at the 1 and 2% level of each silicate; this was significant only with the 2% level of kaolin A. Caloric efficiency followed a similar pattern, except that the 3% kaolin B group was unexpectedly high (40).

Sibbald et al. provided a chick starter diet plus 0, 6, 12, 18, 24, 30, 36 and 42% kaolin to 4 replicated pens of 14 chicks (4 day old). A three day adjustment preriod was followed by a seven day test period during which weight gain and feed consumption were recorded. Weight gain decreased sharply at the 18% level and above; feed consumption was high for all animals consuming kaolin (427).

Day old New Hampshire chicks were fed a glucose-casein diet plus 0 or 5% kaolin for 4 weeks. No ill effects were mentioned (21).

Rats

Groups of 20 hooded rats, 90 g, were fed a standard diet plus 0, 66, or 80% powdered kaolin for 9-12 months. Growth and feed consumption were recorded by Dowling et al. as was fecal weight. After the sacrifice, stomach, small intestine, and colon were weighed and the jejunum and colon analysed histologically. Rats on the 66% and 80% consumed 3 and 4 times the feed as controls did, respectively. Growth was considerably depressed in both experimental groups. The cecum and colon weights increased markedly in the kaolin-fed groups. Glucose transport (intestinal) increased significantly at both dietary levels. The net serosal fluid transfer volume increased significantly for the 80% diet (330).

Kennedy fed 102 male rats (150-450 g) a 50-50 kaolin-basal diet for 14 days. Animals weighing less than 300 g originally maintained their weight. When the diet was diluted to 75% kailon, only animals below 200 g could maintain their original weight (500).

Riecken et al. fed groups of 6 adult female rats 0, 66, or 80%

powdered kaolin in addition to a basal diet. Animals were sacrificed after 2.5, 4, and 12 weeks. No hypertrophy of the small intestine was found (418).

Weinstein and Weiss fed groups of 2-3 rats 12 g of meat plus 0, 2, and 4 g of kaolin daily for 4 weeks. No untoward effects were mentioned (447).

Man

Edwards et al. reported a craving for clay in some pregnant women from rural areas of the southeastern U.S. From 6 to 130 g of clay was consumed daily to achieve a feeling of satisfaction. Several of the women believed that clay relieved the nausea and vomiting common to pregnancy. Diets were low in calories, calcium, iron, thiamine, and niacin. After parturition, the craving disappeared. Two of the 42 women questioned said their babies had a "heavy white covering at birth" (335).

III. Long-Term Studies

A. Bentonite

No Information Available

B. Kaolin

No Information Available

IV. Special Studies

A. Bentonite

Mice

Hepatomus developed in 11 out of 12 mice fed a semisynthetic basal diet mixed with an equal volume of bentonite ("Volclay Bentonite"). The table below describes the number of rats per group, strain, study length, as well as tumor incidence.

Tumor incidence in mice fed the various diets, and controls

Experimental treatment	Strain	No.	Period of treatment (days)	No. with tumors
50/50 Bentonite-basal diet	BUC	10	210-480	9
50/50 Bentonite-basal diet	BUB	2	203-269	2
Controls, basal diet, 51 A	BUC BUB	7 2	211-375 283-294	1 0
50/50 Ruffex-basal diet	BUC	9	280+	0
Thorotrast injections	BUC (varied)	18	216-571	0
Low-protein, choline-deficient diet	BUC BUA	7 2	252-437 414	2 1
BUC mice from stock	BUC	24	484-575	0

The livers of mice on the 50/50 bentonite-basal diet were fibrotic and otherwise severely damaged. Because the liver damage in this group resembled that of mice on a choline deficient diet, it was suggested that bentonite acts as a base exchange silicate in the intestine, removing the nutrient choline as it passes through the intestinal tract (291).

A second paper by Wilson described in more detail the microscopic changes undergone by these eleven mice. Histological examination of these animals revealed fatty livers and some fibrosis with a progressive loss of architecture after 3-4 months. Brownish pigment, apparently ceroid, accumulated. By the 60th day, the size of the nuclei increased, especially in the centrolobular area. The cells enlarged; the cytoplasm was granular and eosinophilic. Livers were described as small. Wilson reiterated his belief that bentonite induced more than a simple choline deficiency (292).

B. Kaolin

Mice

Leu and Ruttner gave groups of 30 inbred male or female mice one intraperitoneal injection of 0.5 ml of a 1% suspension of quartz or kaolin (bolus alba). The animals were sacrificed between 2 and 52 weeks later. Injection of kaolin produced dust cell granulomas with a widening and splitting of the basement membrane of the capillaries. The transformation of the dust cell modules into dust cell granulomas was identicle for the dusts tested (382).

Rabbits

A 37 year old male who had ingested a preparation containing kaolin for a long period of time (unspecified) suffered from abdominal pain, pyrosis, nausea, and vomiting for 8 years. Microscopic studies revealed a few small granulomatous lesions in the prepyloric region (318).

In a subsequent study 4 rabbits were tested with this same preparation (English kaolin colloidal 20% and aluminum hydroxide 2.5% in liquid petrolatum). After a small area of the stomach mucosa had been lightly curetted, the animals were fed a ration containing an unspecified amount of the kaolin. Although no gross mucosal lesions were observed, there were microscopic changes in the submucosa. Multinucleated giant cells occurred singly or in groups, were encapsulated by a fine loose connective tissue, and surrounded by lymphocytes, plasma cells, and eosinophilic neutrophils (318).

Monkeys

Reed et al. issued a report on granulomas caused by faulty esophageal intubation of kaolin preparations in macaques. Sixteen macaques (13 M. mulatta, 2 M. nemestrina, and 1 M. arctoides) from 6 different laboratories developed subcutaneous cervical or mediastinal foreign body granulomas. The cervical lesions were benign. The lesions resulted from penetration of the pharynx or cheek pouch (417).

Man

Potts described two subctuaneous granulomas resulting from the application of kaolin poultices to the breast and coccygeal region of two young women (22-30 yr). Similar lesions were discovered in guinea pigs injected s.c. with an unspecified amount of kaolin (530).

Biochemical Aspects

I. Breakdown

No Information Available

II. Absorption - Distribution

No studies have been performed specifically to examine the absorption of either clay or bentonite. Riecken et al. (418) stated "it is assumed that no absorption takes place during intestinal transit". These authors considered the possibility that absorption of kaolin might occur by pinocytosis. However, the intestinal unspecific acid phosphatase activity should be altered. Since no change in activity was found following kaolin feeding in rats, they concluded that very little if any kaolin was absorbed (418).

III. Metabolism and Excretion

Kaolin is generally considered to be inert (489).

According to Boyd et al. the bulk of orally administered kaolin (100-210 g/kg) was eliminated by rats in the feces during the first two days (310).

IV. Effects on Enzymes and Other Biochemical Parameters

Kaolin has been given orally in doses of 100 g several times a day as an absorbent for intestinal disorders (489).

Bentonite has been used successfully in the treatment of diarrhea. Diarrhea of diverse etiology was relieved in 34 out of 35 cases in an average of 3.8 days. Doses given were 2-tablespoons of bentonite in water 3 times daily. Abdominal cramping, anorexia, malaise, headache, nausea, and weakness were also relieved (38).

In 1923 Braafladt reported that kaolin (1-2 oz/day for man) taken orally for 10-30 days changed the intestinal flora of rats, dogs, and men from a predominantly proteolytic type to an aciduric one. Kaolin eliminated B. welchii from the stool (311).

Olesen tested bentonite for antipeptic activity and the prevention of experimental ulcer formation in Shay rats. Shay described a procedure for the uniform production of gastric ulcers in rats. Starved female Wistar rats underwent pyloric ligation. Then 5 mls of a 5% bentonite suspension or 5 ml distilled water were given by gavage. Twelve to sixteen hours after the operation, ligation of the esophagus was performed and the stomachs removed. Volume of gastric content, pH, free and total

acidity, and pepsin activity were measured. Ulceration was rated as well. Bentonite had a marked protective effect. Pepsin activity was distinctly reduced (524).

Riecken et al. found increased enzyme activity of leucine aminopeptidase in the intestinal mucosa of rats fed 66 and 80% kaolin for up to 12 weeks. The oxidative enzymes demonstrated a similar but not as consistent an increase in activity. These enzymatic changes were associated with an increased intestinal absorptive capacity (418).

Hitchens et al. examined the analgesic action of kaolin in mice and rats subjected to the rat pressure pain test, the phenylquinone writhing test (mice), and the mouse hot plate test. The dosage used was 0.1 ml per 10 g of body weight of a 0-16% solution of kaolin in a 5% gum tragacanth suspension; intraperitoneal, subcutaneous, and oral routes were used for each test. All three routes produced a significant elevation in the pain threshold for the rat pressure pain test, but only the i.p. route was effective in the phenylquinone writhing test and the mouse hot plate test (360).

Coulon et al. used kaolin as an inflammatory agent in rats. A 10% suspension of kaolin was injected into the tarsotibial joint; swelling and skin coloration changes occurred within one hour (319).

Kipiani reported that high concentrations of Askansk clay adversely affected workers at the Makharadze bentonite powder plant. The maximum permissible amount of dust had been exceeded by a factor of 10-100. Ninety-six subjects (packers, millers, operators, filters, and electricians) were found to suffer from bronchospasm, disproteinemia, increased serum neuraminic acid, and reduced phagocytic activity of the leukocytes (120).

V. Drug Interaction

Laughland and Phillips reported that bentonite had an adverse effect on vitamin A metabolism in the rat. Groups of 6 rats (male and female) were fed a vitamin A deficient diet for three weeks, and then fed 0, 0.5, 1.0, 1.5, or 2.5% bentonite. A vitamin A supplement was fed concurrently. After 3 weeks, impaired liver storage of vitamin A was found to be correlated to the amount of bentonite in the diet (137).

An in vitro study by Laughland and Phillips revealed that in addition to the physical absorption of vitamin A on bentonite vitamin A alcohol underwent a dehydration in the presence of sodium bentonite to yield anhydro vitamin A. This anhydro compound formed a blue absorption complex on the bentonite (136).

Brune and Eger studied the interaction of vitamin D_3 (activated 7-dehydrocholesterol) and kaolin on growing rats with chronic vitamin D_3 hypervitaminosis. The femur, tibia, parathyroid, thyroid, pituitary gland, testicles, thymus and kidneys were examined grossly and histologically. Kaolin given orally improved vitamin D_3 resorption (314).

VI. Consumer Explosure

Kaolin is used as an anticaking agent in foods, especially in the production of soybean flour (408). Bentonite has been used as a bonding agent for pelleted feeds (138). Bentonite has been used as a clarification agent for beer, wine, and fruit juices.

BENTONITE AND CLAY

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*Article to be found in text

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MINERALOGY

Far Infra-red Spectra of Montmorillonite, Kaolinite and Illite

ALTHOUGH infra-red spectra of many of the common rock-forming minerals have been presented by several investigators[1-5], few results are available for the diagnostic analysis of clay minerals in the far infra-red spectral region (below 400 cm^{-1}). This communication presents the far infra-red spectra of montmorillonite, kaolinite, and illite for the portion of the spectrum between 90 and 400 cm^{-1}.

Absorption maxima in this region most likely reflect the presence of complex molecular librations and low frequency stretching and bending modes. Inasmuch as relationships between the different clay structures and their far infra-red spectra are essentially unknown, to speculate at length on probable assignments of different absorption bands would be premature. Only qualitative results have so far been obtained.

The samples examined were obtained from the following sources: montmorillonite and kaolinite from American Kaolin Products Co., Aiken, South Carolina; illite (Fithian) and bentonite from American Colloid Co., Skokie, Illinois. Relative purity of the samples was checked by both X-ray diffraction and conventional infra-red methods.

The infra-red patterns for the conventional range are presented in Fig. 1. Referring to these spectra, it is apparent that the samples utilized are of reasonable purity. Fithian illite, even though known to be a mixture of mica, expansible layers, quartz and kaolinite, was chosen because references to it and its characteristics are common in the general literature of clay mineralogy. Bentonite, owing to its usually high montmorillonite content, served as a check on those absorption bands obtained with the 'normal' montmorillonite sample.

An examination of the spectra in Fig. 1 shows that quartz indicated by the characteristic doublet at 800 and 780 cm^{-1} and absorption at 700 cm^{-1}) is a common impurity in all samples. X-ray diffraction studies indicate quartz to be present in amounts less than 10 per cent in all samples. The presence of some kaolinite (<10 per cent as confirmed by X-ray diffraction techniques) is indicated in both the montmorillonite and illite samples. On the basis of X-ray diffraction and infra-red studies, it is felt that the major absorption bands present in the far infra-red region (90–400 cm^{-1}) are likely characteristic of the clay minerals studied and not of the impurities present.

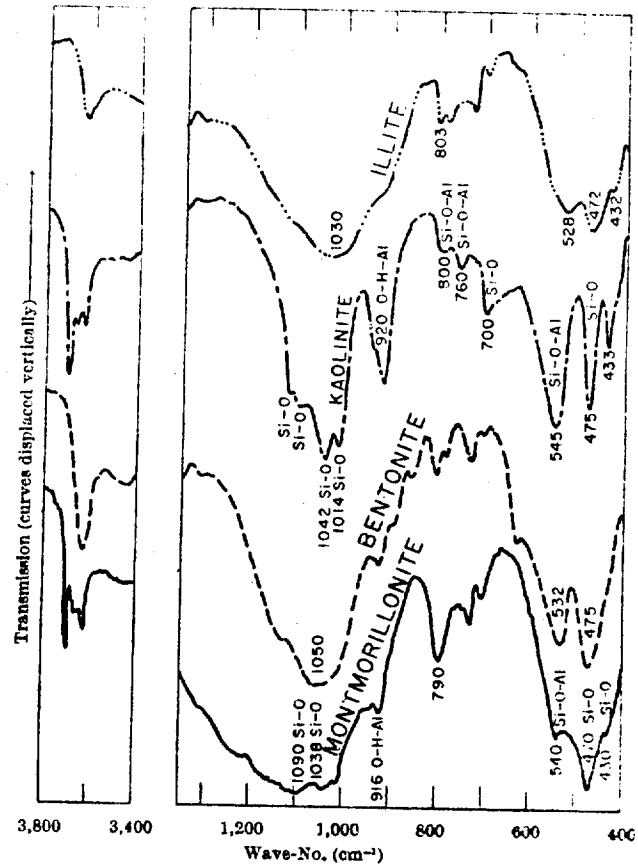

Fig. 1. Spectra of clay minerals investigated (conventional range). Assignment of absorption peaks indicated where known

The samples were prepared by grinding with a mortar and pestle (with ethanol) to a size of 5 microns or less. Mineral oil ('Nujol') was added to form a thick mull (to enhance any weak absorption bands) and a portion of the mull was then pressed between two 0·51 mm thick polyethylene plates. Measurement of the spectra in the range 33–800 cm^{-1} was made on a Beckman *IR*-11 spectrophotometer.

Afterwards, the thick mull was thinned by the addition of more 'Nujol', a portion of this mixture pressed between polyethylene plates and a scan again made from 150 to 800 cm^{-1} (Beckman *IR*-11). This latter step was made to bring the more intense bands 'on scale'. A second portion of the thin mull was pressed between thin (2–3 mm) CsI plates and run in the conventional range (400–4,000 cm^{-1}) on a Beckman *IR*-9 unit.

The spectra of montmorillonite and bentonite and of kaolinite and illite are shown in Figs. 2 and 3 respectively. Assignments (Fig. 1) for the main absorption bands in the 400–600 cm^{-1} region are indicated where known[6]. It is not possible at present to make definite assignments of the absorption peaks beyond 400 cm^{-1}. The primary purpose of this communication is to present the far infra-red spectra of these compounds.

Although possible weak absorption peaks were indicated, the region below 80 cm^{-1} showed more noise than signal to be diagnostic. Absorption in the range 530–555 cm^{-1} (noted in all samples) has been assigned[6] to the Si-O-Al group for montmorillonite and kaolinite; that in bands

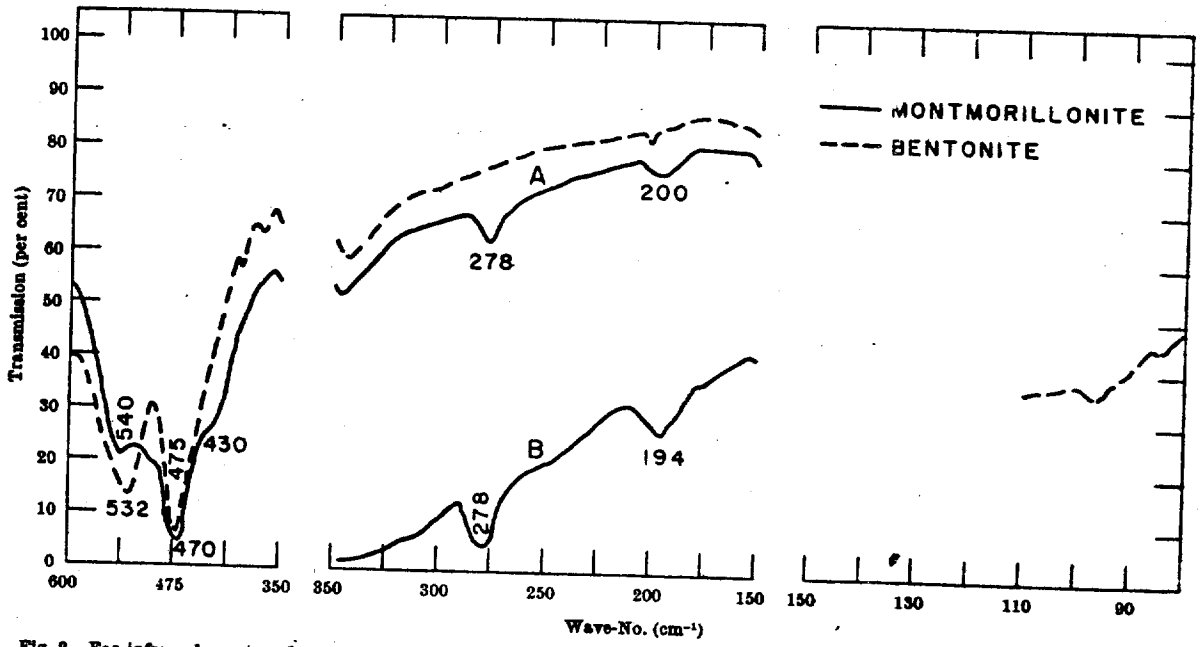

Fig. 2. Far infra-red spectra of montmorillonite and bentonite. Curve *A* and 350–600 cm⁻¹ thin mull between polyethylene plates; curve *B*, thick mull between polyethylene plates

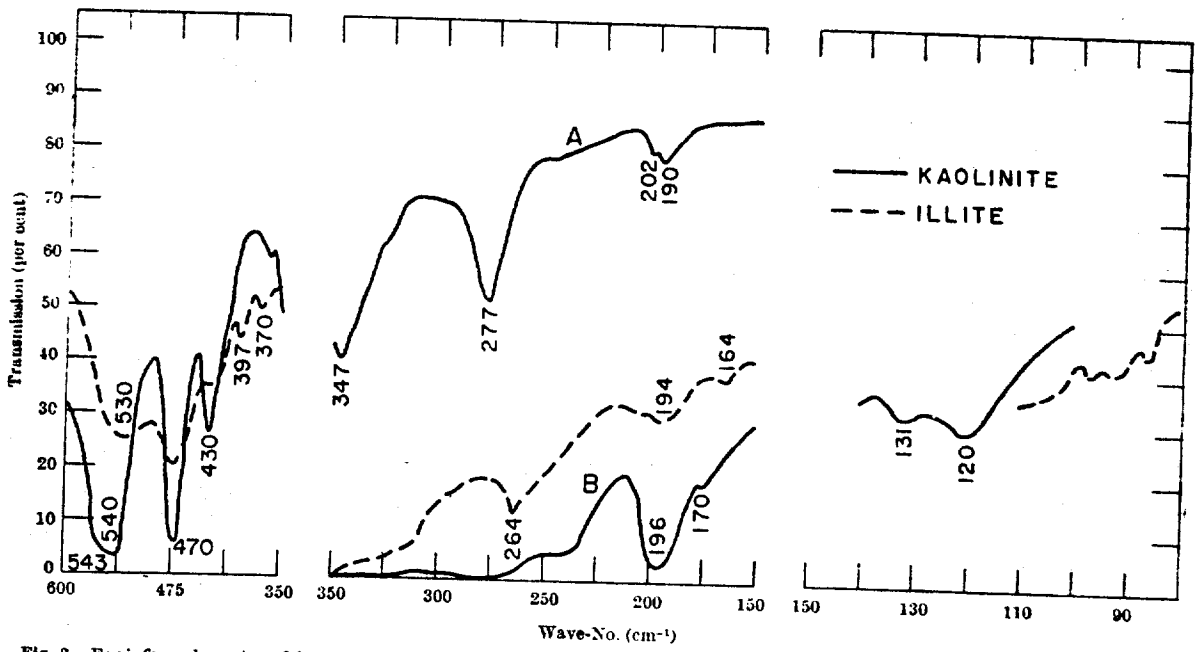

Fig. 3. Far infra-red spectra of kaolinite and illite. Kaolinite, curve *A* and 350–600 cm⁻¹ thin mull; curve *B* and 90–150 cm⁻¹ thick mull. Illite thin mull 350–600 cm⁻¹, thick mull 80–350 cm⁻¹

470–475 cm⁻¹ and 430–433 cm⁻¹ has similarly been assigned to Si-O for the same minerals. It is likely that these vibrational modes in the illite, owing to its compositional make-up, are due to the same atomic groupings. Bentonite, being dominantly montmorillonite, would be expected to show an absorption pattern similar to montmorillonite.

Normally in the range 400–4,000 cm⁻¹, it is difficult to distinguish montmorillonite and illite in the same sample. The identification of these two minerals is commonly based on the presence of strong partially overlapping absorption bands centred at 1,040 and 1,030 cm⁻¹ respectively. Examination of the far infra-red spectra of these two clay minerals reveals the presence of one major and several minor and distinct non-overlapping absorption bands (illite-major 264 cm⁻¹, minor 164, 397 cm⁻¹, etc.) which might allow positive easy identification of these minerals.

Although several of the major bands of kaolinite and montmorillonite are similar, the relatively strong absorption of kaolinite at 120 and 131 cm⁻¹ (these peaks are absent in montmorillonite) should likewise allow positive identification and separation of this pair.

The absorption maxima at 344–348 cm⁻¹ in montmorillonite and kaolinite might be a fundamental vibrational mode, but more likely they are difference bands — in kaolinite 543 − 196 = 347, in montmorillonite 540 − 194 = 346. The band at 194–200 cm⁻¹ was noted in all spectra and although assignment of this peak cannot be made at present, its ubiquitous nature suggests its probable relation to some common structural feature of the phyllosilicates.

At present the far infra-red spectra of clay minerals are of limited usefulness for the purposes of quantitative

analyses. However, their potential value for qualitative work is clear. Investigations of the far infra-red spectra (below 400 cm^{-1}) of the different clays and rock-forming minerals could open a whole new range of possibilities in the investigation of rock-forming compounds.

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ERNEST E. ANGINO

Department of Oceanography and Meteorology,
Texas A and M University,
College Station, Texas.

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Death from Bowel Obstruction Due to Intragastrically Administered Kaolin

• ELDON M. BOYD, M.D. • EARLE L. COVERT, M.D.
• M. N. SHANAS, B.Sc.
Department of Pharmacology
Queen's University
Kingston, Ontario, Canada

This project was undertaken for two reasons. In the first place, in this laboratory, the oral $LD_{50} \pm$ S.E. of powdered iron was found to be 98.6 ± 26.7 g/kg in albino rats and it appeared that some of the deaths could have been due to bowel obstruction.[1] It was decided, therefore, to compare the acute oral toxicity of powdered iron with that of kaolin, a drug held to be inert except for the dangers of bowel obstruction and perforation.[2] In the second place, studies are being undertaken in this department on the acute oral toxicity of items of diet, such as sugar,[3] and their effect upon drug toxicity.[4] Since diet contains a variable amount of silicates, measurement of the acute oral toxicity of kaolin provided an opportunity to assess the significance of this dietary factor.

A review of the literature disclosed no previous publication of the clinical and pathological syndrome of intoxication from kaolin administered orally to albino rats at the range of the median lethal dose. In 1938 it was reported that certain South American[5] and African[6] tribes eat clay, which was found to be mostly kaolin, along with their vegetable diet. A silicosis-like gastric granuloma has been reported in one patient who had used excessive amounts of kaolin for gastric pain, nausea, and vomiting but attempts to reproduce this in rabbits failed unless the kaolin was injected into the gastric mucosa.[7] Mice given a diet of half kaolin develop growth inhibition[8] and hepatomas,[9] due in part to promotion of a choline deficiency. Sollmann[10] referred to work published in 1917 which suggested that excessive oral use of kaolin may lead to its accumulation in the intestines but, he stated, "serious trouble is exceptional." Silica added to the diet in amounts corresponding to an intake of up to 2 g/kg/day has been reported to have no effect upon mice, rats, guinea pigs, rabbits, and monkeys[11] but does increase the silica content of liver, spleen and testes.[12] Feeding silica-containing diatomaceous earth has been found to have no effect upon the silica content of liver and spleen in rats.[13]

From the literature it was concluded that kaolin would probably have no effect given in doses less than about 50 g/kg to albino rats. Pilot experiments were undertaken with doses beginning at 50 g/kg and progressing upward. From experience gained in these preliminary trials, the definitive experiments described below were undertaken.

Methods

The experiments were performed upon male CBL Wistar albino rats fed Purina laboratory chow and water ad libitum. Small animals weighing 90 to 100 g were used, since they had a relatively larger stomach which could adjust better than that of larger rats to the large doses of kaolin which had to be given to produce obstructive deaths. Food, but not water, was withdrawn for 16 hours (overnight) before kaolin administration.

In pilot experiments it was found that a relatively thin suspension of kaolin in distilled water given by stomach tube in the volumes needed to produce obstructive deaths tended to be regurgitated and aspirated into the lungs producing deaths by asphyxia. Regurgitation occurred to a minimal degree with a suspension containing 1 g of Colloidal Kaolin per 1 ml of final volume in distilled water and this suspension was used in subsequent administration. To avoid possible local or systemic effects from cooling, it was heated to body temperature before administration.

Kaolin was administered intragastrically in doses of 100, 110, 120, 140, 145, 150, 155, 160, 175, and 210 g/kg as the required ml/kg of the 100% (w/v) suspension, each dose being given to 12 to 16 rats, with 14 controls given 150 ml/kg

*This project was assisted financially by a grant from the Medical Research Council of Canada.

of distilled water. The suspension was given slowly at the rate of 2 ml/minute to a maximum of not over 120 ml/kg on the first administration. Administration of larger volumes at one time produced gastric perforation in practically all animals. When a second administration had to be given, an interval of three hours was allowed to elapse. This was the shortest interval necessary to reduce the incidence of gastric perforation from the second administration. Of 140 rats so treated, 47 were found to have died from aspiration or gastric rupture and were discarded, leaving 93 upon which the following observations and measurements were made.

Clinical measurements were made upon each rat in its metabolism cage at daily, or shorter if indicated, intervals for five days. They were then observed to the end of one month. The measurements made were body weight in g, food consumption in g chow/kg body weight/24 hours, water consumption in ml/kg/24 hours, colonic temperature in °F., urinary volume in ml/kg/24 hours, urinary glucose output in mg/kg/24 hours, urinary protein output in mg/kg/24 hours, urinary pH, and other clinical signs as they appeared and which were semi-quantitated as 1+ to 4+. Colonic temperature was recorded by a Thermistemp Telethermometer with the probe inserted 8 to 10 cm per rectum. Urinary glucose, protein, and pH were estimated with Ames Combistix Reagent strips. The rate of elimination of kaolin in feces was estimated from the number of white fecal pellets excreted per day and in total.

Gross pathology was recorded at autopsy on all animals which died. Determinations of the fresh wet weight and water level of organs were made upon animals which could be autopsied within one hour of death to avoid postmortal shifts reported from this laboratory.[14] This was accomplished upon rats given an ED_{50}* of kaolin and killed with chloroform 48 hours later, data being assembled upon 12 surviving animals and 12 controls. Weights and water levels were also measured upon 12 survivors and 12 controls at each of two weeks and one month. Wet weight was measured to 0.0005 g upon a Gram-atic Semi Micro Balance. The organs examined are listed in Tables I and II. The sample of skeletal muscle was the left half of the ventral abdominal wall muscle layer. The contents of the gastrointestinal tract were removed by a standardized method of washing and milking before each weighing. Water levels were measured upon the same organs by drying a weighed aliquot to constant weight at 95°C. in a Fisher Forced Draft Isotemp Oven. The aliquot of skin taken for water analysis was from the dorsolumbar region. The residual carcass, after removing the various organs, was cut into small pieces, homogenized in a Waring blendor and an aliquot selected for water analysis. Water levels were calculated as g water per 100 g dry weight of tissue. Histopathologic examinations were made upon blocks of tissue fixed in Lillie's buffered formalin with sections stained with hematoxylin-phloxine-saffron. Statistical methods were those of Croxton.[15]

*ED_{50} as used in this report is the dose which killed 50% of animals by bowel obstruction. It is equivalent to the LD_{50} due to bowel obstruction.

Results

The $ED_{50} \pm$ S.E. was found to be 148.5 ± 16.8 g/kg. An estimate of the ED_1 was 110 ± 17 g/kg and of the ED_{100} 188 ± 17 g/kg. The mean ± standard deviation hours to death was 43.3 ± 15.4 in 40 rats that survived death

TABLE I
SHIFTS IN THE WET WEIGHT OF ORGANS AT AUTOPSY[1]

Organ	At 2 Days	Survivors at 2 Weeks	Survivors at 1 Month
Adrenal glands	+19.2*	− 7.9	+ 2.3
Brain	+ 3.6	− 0.6	− 0.6
Gastrointestinal tract			
a) Cardiac stomach	+16.6*	+ 0.1	− 0.2
b) Pyloric stomach	+ 4.8	− 0.1	−10.5
c) Small bowel	+37.1**	− 4.8	+18.1
d) Cecum	−12.5	+10.2	− 2.0
e) Colon	+ 1.1	−14.0*	− 2.7
Heart	− 5.3	− 5.5	− 2.2
Kidneys	− 4.3	− 6.2	0.0
Liver	− 8.3	− 9.1	+ 8.6
Lungs	− 5.0	−15.5**	−20.0*
Muscle (abd. wall)		−17.8	+ 9.2
Skin	− 9.4**	− 6.8	+ 0.2
Spleen	−24.3*	− 6.7	−21.5*
Testes	+10.9	+ 6.0	− 8.2
Thymus gland	−28.1*	+ 2.2	+30.2*
Residual carcass	− 8.1**	− 6.4	− 5.3

[1]Weight was measured as gm wet weight. The results are expressed as a percent change from controls, specifically as $(|\bar{X}d-\bar{X}c|/\bar{X}c) \times 100$, where Xd is the mean in the drug-treated rats and Xc in the controls. Differences bewteen Xd and Xc significant at P = 0.05 to 0.02 are indicated by one asterisk and at P = 0.01 or less by two asterisks.

TABLE II
SHIFTS IN THE WATER LEVELS OF ORGANS AT AUTOPSY[1]

Organ	At 2 Days	At 2 Weeks	At 1 Month
Adrenal glands	+33.3**	+ 0.4	+ 0.4
Brain	+ 0.8	− 1.1	+ 1.4
Gastrointestinal tract			
a) Cardiac stomach	+17.6**	− 0.1	+ 0.1
b) Pyloric stomach	+ 6.3	+ 3.8	− 4.3
c) Small Bowel	+29.2**	+ 3.7	+ 3.6
d) Cecum	+ 9.2**	+ 3.7	+ 2.7
e) Colon	+12.9	− 3.6	− 6.8
Heart	+ 0.8	+ 0.8	− 0.3
Kidneys	− 0.3	+ 0.6	+ 5.7
Liver	+ 8.9	+10.8*	+ 2.4
Lungs	+ 1.9	+ 1.9	− 0.8
Muscle (abd. wall)	+ 7.2	+18.8*	− 7.8
Skin	+17.8**	− 5.9	− 4.7
Spleen	+14.8**	+ 0.1	+ 3.4
Testes	+ 0.8	− 1.9	− 3.5
Thymus gland	+20.2**	+ 6.2	+ 0.6
Residual carcass	− 2.8	+ 6.2	+11.7*

[1]Water levels were measured at gm water per 100 gm dry weight of tissue. The results are expressed as percent change from controls as was organ weight in Table I.

from aspiration of kaolin into the lungs or gastric perforation and subsequently died from bowel obstruction. The time to death was not dose-dependent within the range of doses studied.

During kaolin administration, the animal went through motions somewhat like those of gagging and retching. As the stomach distended (Figure 1), the animal became tense and there followed a series of abdominal muscular fasciculations beginning at the pubis and passing craniad with some extension to other skeletal muscle. Toward the end of kaolin administration, some rats died suddenly from aspiration into the lungs of regurgitated kaolin; these animals were discarded. Also discarded were rats found dead, usually at 24 hours, and in which the stomach was noted to have been perforated by too rapid administration of kaolin.

Following drug administration, 90% of the animals were listless and withdrawn (failed to respond to stimuli such as sound, prodding, and blowing against the grain of the hair) and had pallor of the ears and tail. Half the rats had convulsive movements within the first five hours.

At 24 to 48 hours, the rats remained pale, drowsy and listless, the paws were dry and 22% developed blood-tinged diarrhea. There was a brownish incrustation about the nose and mouth in all rats at 24 hours and in 50% at 48 hours. Food intake, water intake, body weight gain and

Figure 1
An illustration of gastric distension following administration of kaolin in the range of the ED_{50}.

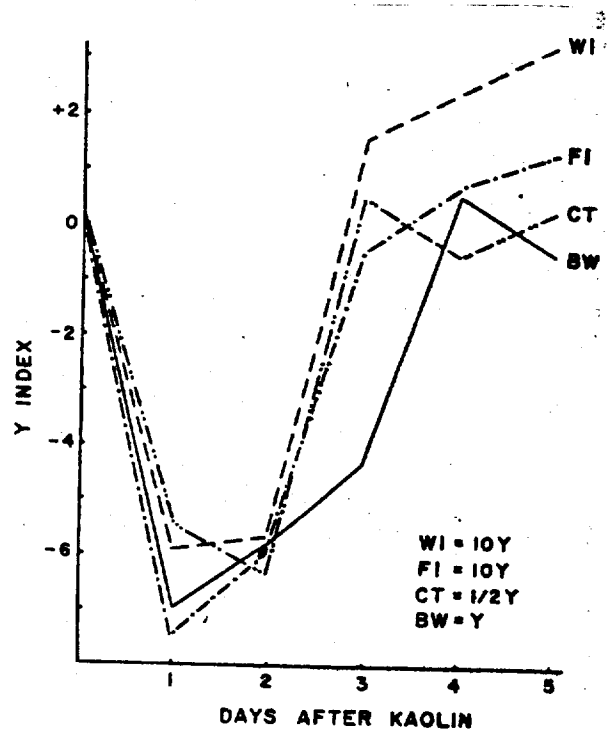

Figure 2
The regression, on days after administration of kaolin, of percentage changes in body weight (BW), food intake (FI), water intake (WI), and colonic temperature (CT). Percentage changes were calculated as $([\bar{X}d-\bar{X}c]/\bar{X}c) \times 100$ where $\bar{X}d$ was the mean in the drug-treated group and $\bar{X}c$ in the controls. The Y Index is multiplied by the indicated factors to obtain the respective percentage changes.

colonic temperature were markedly depressed as illustrated in Figure 2. The estimated weight of retained kaolin was subtracted in arriving at values for body weight. At 24 hours, urinary volume was markedly less than that of the controls (which had a diuresis from the water administration) and there occurred a marked glycosuria and slight aciduria as shown in Figure 3. As indicated in Figure 4, the bulk of administered kaolin was eliminated in feces during the first two days, mostly by rats which subsequently recovered. None of these parameters were dose-dependent within the range of doses studied.

The premortal clinical signs were increasing listlessness, persistence of the brownish incrustation about the mouth and nose, anorexia, oligodipsia, body weight loss, oliguria or anuria, proteinuria, glycosuria, hypothermia, dyspnea and death from respiratory failure.

Bowel obstruction was the dominant feature of gross examination at autopsy. In the small bowel kaolin was impacted in hard, crescent shaped masses at intervals along the intestine as shown in Figure 5. In the cecum and colon, kaolin appeared as a solid mass as illustrated in Figure 6. The only dose-dependent sign, apart from death,

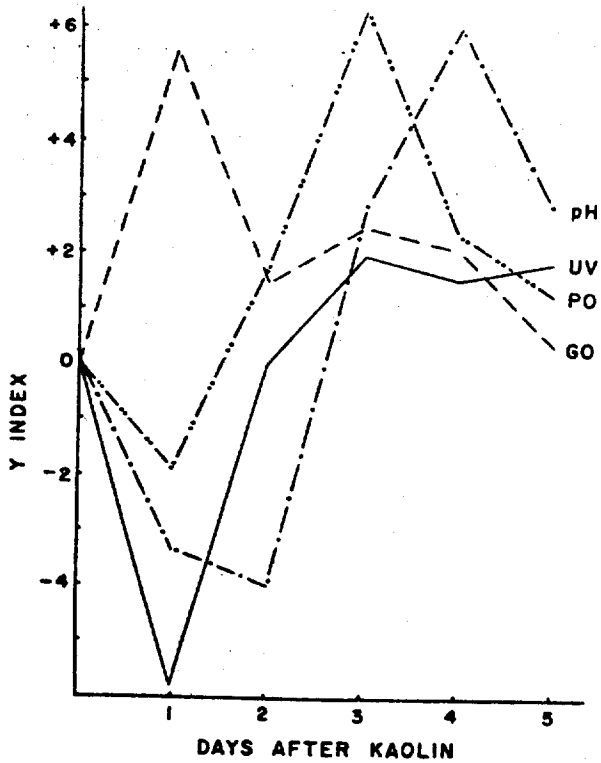

Figure 3
The regression, on days after administration of kaolin, of percentage changes in urinary volume (UV), urinary glucose output (GO), urinary protein output (PO) and urinary pH (pH). Percentage changes are calculated as indicated in the legend for Figure 2. The Y Index is multiplied by three to obtain percentage changes in pH, by 20 for UV, by 60 for PO, and by 1,000 for GO.

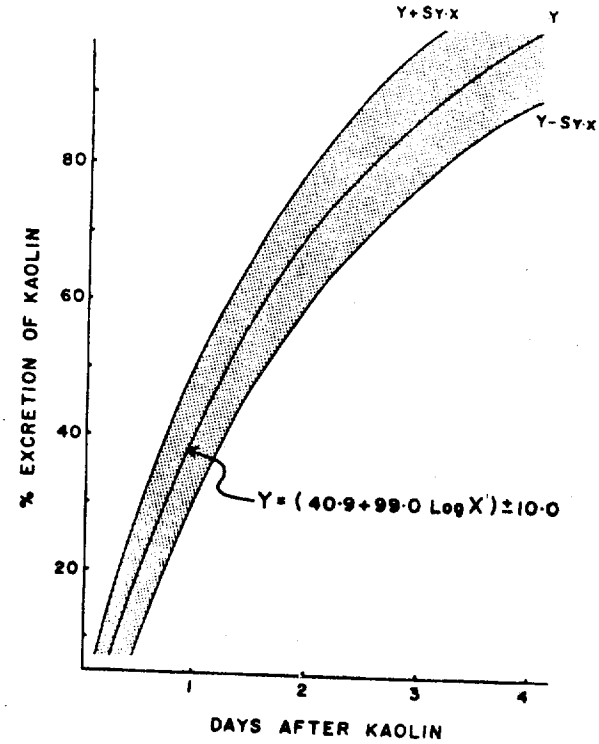

Figure 4
The regression, on days after administration, of the rate of elimination of kaolin in feces.

was the distance kaolin had passed through the small bowel before impaction and death occurred. As indicated in Figure 7, the greater the dose of kaolin the further the drug had advanced through the small bowel.

Other features of gross pathology were enlarged and congested adrenal glands and destruction and atrophy of the thymus gland, congestion and hemorrhage in all parts of the gastrointestinal tract and a small, pale liver and spleen.

Data upon the wet weights of organs at 48 hours are summarized in Table I and upon water levels in Table II. There was a significant increase in the wet weight of adrenal glands and of parts of the gastrointestinal tract due largely to an increase in water content. Loss of wet weight in the skin, spleen, and thymus gland was associated with significant increases in water content, indicating an even greater loss of dry weight in these organs. Since no organ had a significant loss of water and since the animals drank very little water, the increase in water levels must have been due to water administered with the kaolin and not eliminated in urine. This may have been combined with some redistribution of water as suggested, but not proven, by the statistically insignificant mean loss of water in residual carcass.

Histopathologically, when kaolin was impacted in the stomach, the stratified squamous epithelium of cardiac stomach was stretched and necrotic. There was a marked capillary and venous congestion of the lamina propria and submucosa with leucocytic infiltration of all layers, including the

Figure 5
A photograph showing impaction of kaolin in the small intestine.

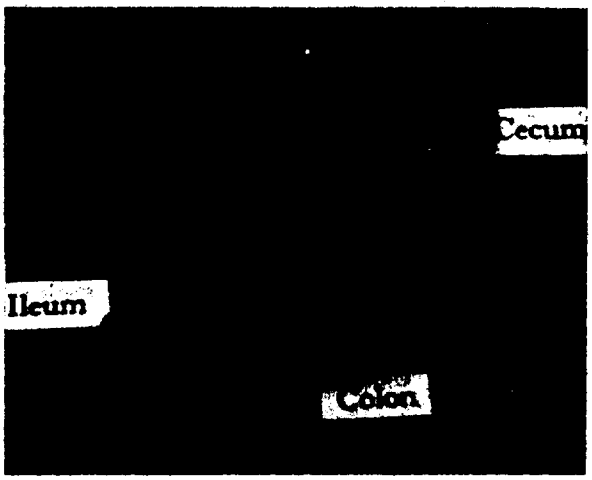

Figure 6
An illustration of kaolin impacted in the cecum and colon.

subserosa. The gastric glands of the pyloric stomach were relatively intact with areas of ulcer-like necrosis, marked capillary-venous congestion and leucocytic infiltration especially of the muscularis externa and serous layer. The cardiac stratified squamous epithelium and the muscularis externa were particularly susceptible to the dilating effect of the large volume of administered kaolin suspension. As kaolin left the stomach, the lining tissues quickly restored themselves under a blanket of mucus, leaving scattered residual ulcers in both parts of the stomach.

At areas of impaction in the small bowel, the wall was distended and thin and the villi were pressed against the wall. In some animals, the villi were denuded to a varying degree but in general there was comparatively little loss of villi. The wall was blanched and avascular over the center of the impacting pellet, but at each end of it there was a marked capillary-venous congestion and there was some hemorrhage into the lumen.

As the pellet passed down the intestine, it acquired a coating of mucus. Behind the obstruction, the intestinal mucosa quickly restored itself. Ahead of the obstruction, the small bowel appeared normal. There was a generalized vasodilatation of mesenteric vessels.

Distension of the cecum by kaolin produced a flattening of the wall and some congestion of the submucosa but little damage to the cecal glands. Kaolin pellets in the colon were covered with mucus, the lumen of the colon contained cellular debris, but the glands of Lieberkuhn were normal and congestion, if present, was very mild.

Other histopathology was of relatively minor significance. The adrenals were hypertrophied with sinusoidal and venous congestion and the thymus was atrophied. The cytoplasm of the hepatic cells was shrunken. The spleen was relatively avascular and the red pulp was shrunken. The renal glomeruli were relatively avascular and occasionally there were areas of cloudy swelling in the tubules. The pulmonary alveolar wall was shrunken and avascular. Venous stasis and thrombosis were seen in liver, adrenals, kidneys, testes, heart, lungs, and brain.

Histopathologically, the cause of death appeared to be circulatory stasis secondary to bowel obstruction. Circulatory stasis apparently led to respiratory failure and death.

Signs of recovery appeared in survivors on the second and third day when they had eliminated most of the kaolin in their feces. The listlessness disappeared and was replaced in 35% of survivors by an aggressive hyperkinesia in which, for example, the animal would attack and bite a probe introduced into his cage. Food intake, water intake, body weight and colonic temperature rose to or above control levels (Figure 2). The glycosuria gradually disappeared but there was a temporary proteinuria, alkalinuria, and polyuria (Figure 3). Organ weights and water levels gradually returned toward control values, but there were a few statistically significant differences at two weeks and even at one month after drug administration (Tables I and II).

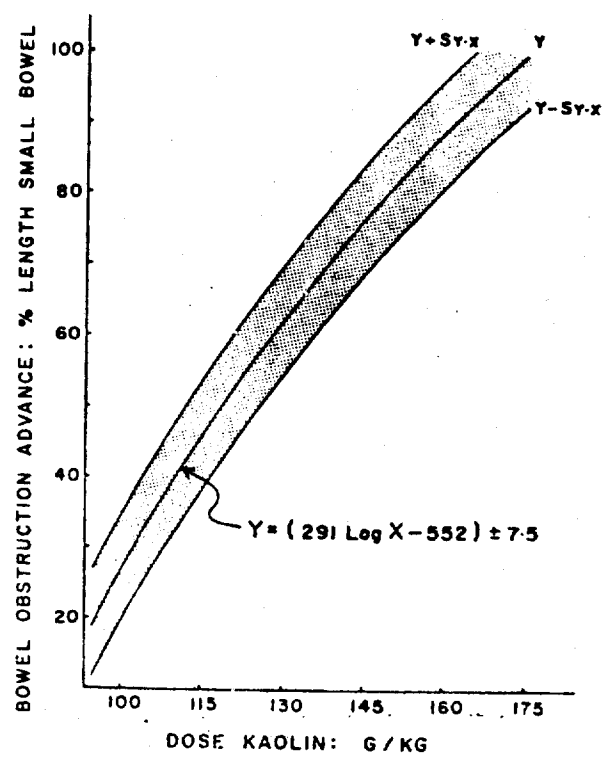

Figure 7
The regression, on dose of kaolin, of the advance of bowel obstruction measured as percentage of the total length of small intestine.

Discussion

Ferguson[16] has noted that death rates from oral administration of some 12 drugs were higher the greater the vol kg of distilled water used as a vehicle. Had it been possible to maintain constant the vol kg of suspended kaolin, and if the relationship established by Ferguson[16] applied to suspended kaolin, the ED_{50} would have been lower than that found. It was not possible to check Ferguson's[16] relationship because, had volume been kept constant at say 200 ml/kg, diluting the suspension below 1 g kaolin per 1 ml suspension would have lead to regurgitation and death from aspiration into the lung.

The largest volume of suspension used in our experiments was 210 ml/kg. In pilot tests, we have found that this volume is about one-half the LD_{50} of distilled water given intragastrically to overnight starved albino rats at intervals similar to those used for the kaolin suspension. Distilled water produced convulsions, respiratory failure and death within a few hours after administration of a lethal dose. The convulsive movements noted following kaolin suspension could have been due to absorption of distilled water, but death from kaolin occurred long after that from distilled water. Summation of sublethal toxicity of water and kaolin cannot be entirely excluded, however, as a factor in our project.

Deaths from aspiration into the lung and from gastric perforation were excluded in arriving at the ED_{50} of kaolin. Is this justified? Within the context of our method we considered that it was. Deaths from aspiration varied with concentration of suspension rather than with g of kaolin. For example, a dose of 100 g/kg of kaolin produced one death from bowel obstruction in 16 rats when given as a suspension containing 1 g/1 ml but when diluted to 0.5 g/1 ml, almost all rats died from aspiration asphyxia. Similarly death from gastric rupture was associated to a greater extent with the volume of suspension and the rate and force of intragastric administration than with the total dose of kaolin. When the rate and force of administration were standardized in a series of preliminary experiments upon a 75% (w/v) suspension given at one time to young rats, the LD_{50} for perforation deaths was approximately 150 ml/kg or about 110 g/kg of kaolin. In older rats, with a smaller ratio of stomach size to body weight, this vol/kg caused gastric perforation in all animals. Since aspiration and perforation deaths were excluded, the ED_{50} established in this project, therefore, was basically the ED_{50} causing death by bowel obstruction.

Lethal bowel obstruction proved to be an all-or-none type of toxicity reaction. Over the range of doses of kaolin studied, increasing doses produced an increasing incidence of lethal bowel obstruction and at increasing distances down the small bowel. Otherwise, however, the toxic reaction was not dose-dependent. Anorexia, for example, was not greater the greater the dose of kaolin. Anorexia and other signs of toxicity are usually dose-dependent in most acute oral toxicity studies. Drugs used in these studies are usually absorbed from the gastrointestinal tract and produce dose-dependent degrees of hepatitis or nephritis or encephalitis or other such reactions.

The results suggest that the amount of kaolin absorbed and the reaction to its absorption bore little or no relation to the dose of kaolin administered — within the range of doses used. The signs of toxicity were apparently due to pathological reaction from over distension of the alimentary canal by an inert solid substance. This pathological reaction to distension was as marked following a dose of 100 g/kg of kaolin as following 210 g/kg except that the number of fatalities and the incidence and advance of bowel obstruction along the small intestine were dose related.

A lack of dose-response relationship was also seen in bowel obstruction produced by intragastric administration of powdered iron — Reduced Iron N.F. IX.[1] In the latter study, however, the interval to death was shorter the greater the dose of iron and there was vascular congestion in many organs including the kidneys, lungs and brain. It would appear, therefore, that reaction to absorbed iron played some role in the syndrome. Pilot studies have indicated that an appreciable amount of iron is absorbed by the inflamed gastrointestinal mucosa when these large doses of powdered iron are administered.

Bowel obstruction produced by powdered iron and by kaolin had certain features in common. These features could represent the pathological reaction to marked distension of the bowel and impaired ability to propel an impacted mass. The features in common were (a) the large obstructing dose (10% of body weight for iron, 15% for kaolin), (b) death delayed to two or more days, (c) listlessness, (d) anorexia, (e) oligodipsia, (f) inhibition of growth, (g) hypothermia, (h) oliguria, and (i) a marked stressor reaction in the adrenal and thymus glands.

Bowel obstruction in man is almost always due to narrowing of the lumen from external causes. The diagnostic signs of complete obstruction are feces vomiting, abdominal pain, cardiovascular shock and constipation. Projectile vomiting does not occur in the rat, but regurgitation was regularly seen when the kaolin suspension was sufficiently dilute. There was no painful phonation

in any of these animals. Constipation was present when bowel obstruction had set in. Points (c) to (h) above could have been due to surgical shock.

Summary

Intragastric administration of kaolin suspensions produced death by regurgitation and aspiration asphyxia, by gastric rupture, and by bowel obstruction in young male albino rats. Death from aspiration and gastric perforation varied with technique and was not studied further. The $ED_{50} \pm$ S.E. producing death from bowel obstruction was 149 $\pm$ 17 g/kg. The interval to death from bowel obstruction was 43 $\pm$ 15 hours. The clinical signs of intoxication were listlessness, pallor, perinasobuccal incrustation, anorexia, oligodipsia, hypothermia, inhibition of growth, oliguria, glycosuria, proteinuria, and aciduria. None of the clinical signs was dose-dependent. At autopsy, bowel obstruction was further advanced along the small bowel the greater the dose of kaolin. There was a significant gain in weight of most parts of the gastrointestinal tract, a loss of weight in skin and spleen, a stressor response in the adrenal and thymus glands and water retention in several organs. When kaolin was impacted in the stomach, it produced necrosis particularly of the stratified squamous epithelium and muscularis externa along with marked inflammation. As kaolin entered the intestine, it became impacted in pellets which acquired a mucous coating and produced less necrosis the further they advanced. Behind the obstruction, the mucosa was quickly restored under a blanket of mucus. The spleen, kidneys, and lungs were hypovascular and venous stasis was present in many organs. The cause of death was respiratory failure following circulatory stasis induced by bowel obstruction. These signs began to disappear in survivors as kaolin was eliminated in feces and autopsy at two weeks and one month revealed few or no signs of residual toxicity.

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THE EFFECT OF KAOLIN ON THE INTESTINAL FLORA IN NORMAL AND PATHOLOGIC CONDITIONS

ONE PLATE

LOUIS HENRY BRAAFLADT

From the John McCormick Institute for Infectious Diseases, Chicago

This study was begun in August, 1919,[1] when the epidemic of Asiatic cholera which swept over China that year reached Tsinan. Kaolin was used with success on a fairly representative number of cases treated at the University Hospital at that time, as well as on a few sporadic cases in 1920. Its effect on acute amebic dysentery, acute bacillary dysentery, and chronic ulcerative colitis was also noted.

Very little work has been done which throws any light on how it affects pathogenic intestinal micro-organisms and their toxins and toxic products. So far is I know, no observations have been recorded dealing with its effect on the normal flora of the intestine.

History of Kaolin.—The etymology of the word "kaolin" is simple, yet interesting. It is a transliteration of two Chinese character which mean high mountain range. When Chinese pronounce these characters, it sounds to a foreigner like kao-ling or gao-ling.

According to Keenan,[2] who has written an interesting history on kaolin, the first appearance of the word in Europe was in a letter from a Jesuit missionary to China, Father Dentrecolle, to the procureur of the order of Paris, in 1712. In this letter, as well as in one written in 1722, he describes in a fascinating way the large porcelain industry about the city King-te-chen, where as many as a half a million people were employed in it. The 3,000 kilns at night so illuminated the hills surrounding the city that it seemed like a vast city abandoned to the flames. Both letters are to be found in the appendix to Bushnell's[3] translation of the classical work on pottery and porcelain, the "T'ao Shuo."

Following the publication of these letters, a contemporary chemist, R. A. F. de Reamur, analyzed specimens of kaolin which had been sent him from China and found them to be the chief ingredient of porcelain or chinaware of the Orient. This seems to have stimulated the search for deposits in France, England, and America.

William Cookworthy discovered deposits at Tregonnin Hill, Cornwall, in 1750. In 1768, he procured a patent for the making of porcelain. Keenan says: "In France the kaolin quarries that made Limoges famous were the accidental

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[1] Editorial, China Med. Jour., 33, p. 574.
[2] American Druggist, 1914, 62, p. 55.
[3] Clarendon Press, Oxford, 1910.

discovery, in 1765, of Madame Darnet, the wife of a surgeon at St. Yrieix, near Limoges. Coming upon a white unctuous earth in a ravine, she thought of using it for washing linen. Having showed it to her husband, he suspected that this might be the clay that all were looking for. He took it to a pharmacist at Bordeaux, named Villaris, who recognized it to be kaolin. This discovery led to the establishment of the remarkable works of Limoges. . . . the principle factory being a purely American concern, The Haviland, and to this day the United States is chiefly supplied with porcelain from the American works of Haviland and of Straus in Limoges."

In this country, the Cherokee Indians are believed to have mined a crude kaolin in North Carolina for export to England more than two centuries ago. "The Indians are said to have carried the clay from the Smoky Mountains to the coast under the name of *Unakah,* which was their name for the Smokies (meaning white), still called Unakah in Mitchell County and Unakoi in Cherokee. A patent was applied for in England in 1744 for the production of porcelain from an earth mixture called Unaker, referred to as 'the produce of the Cherokee nation of America', the name being derived in a manner analogous to that of kaolin in China, from the place of origin of the clay—the mountains bounding North Carolina on the northwest, called Unaker." Watts[4] does not believe that pure kaolin was produced in America as early as this but that it was a mixture of kaolin, feldspar and quartz. It is now mined in Pennsylvania, Delaware, Virginia, Georgia, Florida and North and South Carolina. The total yield in the United States in 1912 was only 27,000 tons. The deposits in Cornwall and Devon, England, are still being worked on a large scale. In 1912, 237,366 tons of English clay valued at $1,541,105 were imported by this country.

Its Composition.—Feldspar of granitic rock is a double silicate of aluminum and alkali. When exposed to the prolonged action of water charged with carbon dioxide, weathering takes place, that is, the feldspar breaks down. The alkali is eliminated and water is taken up, the resulting substance being hydrous aluminum silicate. Keenan represents this action as follows:

Feldspar.....	K_2O	Al_2O_3	$6 \cdot Si\ O_2$	
Removal of.....	K_2O		$4\ Si\ O_2$	
Addition of.....				$2\ H_2O$
		Al_2O_3	$2\ Si\ O_2$	$2\ H_2O$

Rains and floods have washed this decomposition product to places where it has become mixed with quartz, sand and various minerals. Some of the purest deposits of kaolin in America are the result of sedimentation during times of floods.

The crude kaolin when dug from its bed or washed out hydraulically is usually purified, that is, freed from gritty particles, such as sand and mica, by repeated washing and decantation (elutriation). It is then run into filter presses where the water, at a pressure of 100 to 120 pounds per square inch, escapes. The moist cakes are left to dry by exposing them to the air or steam heat. This product contains more or less impurities, depending on the thoroughness of the washing and the efficiency of the method used to remove the water. For pharmaceutical purposes, the alkali which it may contain is removed by treating with 5% hydrochloric acid. After that it is treated with dilute sulphuric acid to remove other impurities, and then washed thoroughly with water to remove the chlorides and sulphates. A pure kaolin contains approximately 47% of silica, 40% of aluminia, and 13% of water.[5]

[4] Bull. 53, Dept. of Interior, Bureau of Mines, 1913, p. 37.
[5] U. S. Dispensatory, Ed. 20, p. 605.

Its Properties.—The British Pharmacopoeia[6] describes it as "a soft whitish powder, insoluble in water, or in dilute acids." It was introduced into the U. S. P. VIII, for the purpose of forming the basis of the preparation of Cataplasma Kaolini, which was dropped from the U. S. P. IX, but introduced into the National Formulary, Ed. IV.[7] Here it is described as "a soft, white, or yellowish white powder or in lumps, insoluble in water and in cold dilute acids and in solutions of the alkali hydroxides. It has an earthy or clay-like odor when moistened with water." Tests for its purity are found in the U. S. P. VIII, the B. P., and other pharmacopeias.

Flack,[8] experimenting on several samples of kaolin from reliable sources, found that they did not possess similar amounts of adsorptive power. Scoville[9] showed that alkaloids present in solutions filtered through a layer of kaolin are removed by it. Fantus,[10] correctly, I think, maintains that Fuller's earth should not be given as a synonym for kaolin because of differences in their adsorptive action.

The pharmacopeias of Britain,[11] Norway,[12] and the United States (VIII[13]) describe kaolin under its proper name. Those of Germany,[14] Austria,[15] Belgium,[16] The Netherlands,[17] Japan,[18] and Italy[19] fail to distinguish kaolin from the argillaceus earths which have been known for ages as "bolus," "bolus alba" being the term under which they describe it.

Its Uses.—These are manifold. It is used in the manufacture of porcelain, pottery and false teeth; in certain toilet powders and face cleansing clays (popular today, and selling at high prices; when a thick suspension of kaolin in water, costing about 30 cents a pound would probably be just as effective). It is used as filtering medium to clear syrup, beer and cloudy solutions of volatile oils. In pharmacy and medicine its chief use has been as a basis of a cleanly form of poultice, "cataplasma kaolini," N. F.; as an excipient in making pills which are readily oxidized or reduced when in contact with organic matter, especially pills of potassium permanganate, silver nitrate, silver oxide and the like. It has been used as a dusting powder in wounds on ulcerated surfaces and diphtheritic sore throats. Hemmeter, many years ago, advised its use in hyperacidity of the stomach.

The success with which it has been used during the last few years in gastro-intestinal disorders produced by bacteria that liberate soluble toxic products in the intestine, would seem to justify amply the time spent on this study.

Owing to differences observed by Scoville and Fantus in particular, in the adsorptive action of various specimens on the market, the necessity for using a standardized, pure product in internal disorders and in experimental work to obtain uniform and reliable results, is apparent. That used throughout this work was Merck & Company's: Kaolin Merck, U. S. P. VIII.

[6] The British Pharmocopoeia, 1914, p. 199.
[7] The National Formulary, Ed. 4, 1916, p. 309.
[8] Am. Jour. Pharm., 1906, 78, p. 419.
[9] Ibid., 1916, 88, p. 506.
[10] Jour. Am. Med. Assn., 1915, 64, p. 1838.
[11] The British Pharmaceutical Codex, 1911, p. 556.
[12] Den Norske Pharmacopœ, Ed. IV, 1913, p. 183.
[13] U. S. P. VIII Revision, 1905, p. 252.
[14] Pharmacopeia Germanica, Ed. Altera, 1882, p. 42.
[15] Pharmocopeia Austria, Edito Octava, 1906, p. 60.
[16] Pharmacopeia Belgica, Ed. II, 1885, p. 40.
[17] Nederlandsche Pharmacopoe, IV, 1915, p. 67.
[18] The Pharmacopœia of Japan, III, 1907, p. 60.
[19] Pharmacopeia Ufficiale del Regno D'Italia, IV, 1920, p. 71.

EXPERIMENTAL WORK

This was conducted on rats, rabbits, guinea-pigs, dogs, and on 4 healthy men volunteers. It was hoped that it would throw some light on:

(*a*) The effect that kaolin has on pathogenic organisms that invade the intestine of man, such as the dysentery bacillus, typhoid and paratyphoid bacilli, B. enteritidis, B. botulinus, and Vib. cholerae.

(*b*) Its effect on the toxins and toxic products produced by these organisms.

(*c*) Its effect on the normal flora of the intestine.

The Effect of Kaolin on Bacteria Growing in Artificial Mediums.—One gram of sterile kaolin was added to 24-hour cultures (about 8 c c. of favorable medium in each tube) of B. dysenteriae (Shiga), *B.* typhosus and B. paratyphosus, B. botulinus, B. enteritidis. The cultures were left at room temperature for two hours, being shaken up well every half hour. In subcultures growth was obtained from all of them.

Although it is evident from this that kaolin is not strongly bactericidal, in agreement with Walker [20] and others, still there is a possibility that it might inhibit the growth of bacteria or accelerate it.

Culver and Novrogordato [21] found that certain insoluble and chemically inert dusts, such as quartzite and wood charcoal, added to culture medium augmented the growth of B. pyocyaneous, streptococcus, and B. tuberculosis (human).

Two experiments were made to ascertain whether kaolin has a similar effect:

1. One c c. of a suspension of B. coli was added to 5 c c. of sterile salt solution; further dilutions were made from this into other 5 c c. amounts. Agar tubes, each containing exactly 5 c c. of medium, were melted. To each of half the number of agar tubes, 10 mg. of sterile kaolin were added, also 0.1 c c. of B. coli dilution. They were shaken well and plated. After 24 hour's incubation, the number of colonies on the kaolinized plates that could be counted with accuracy, using the low power lens, were compared with the numbers on the corresponding control plates. The difference between the average number obtained from 3 plates and their controls were negligible.

2. In the second experiment, 1 gm. of kaolin was added to a tube containing 8 c c. of broth. This was inoculated with 0.1 c c. of a 24-hour broth culture of B. coli. A control tube containing a similar amount of broth was inoculated with 0.1 c c. of the same culture. The tubes were incubated for 3 hours, being shaken up thoroughly every 5 minutes. Then 0.1 c c. from each tube was added to 2 tubes containing 5 c c. of sterile salt solution; further dilutions were made

[20] Proc. Royal Soc. of Med., 1921, 14, p. 23.
[21] Med. Jour. of South Africa, 17, No. 2, p. 209.

from these. (The kaolinized culture, as well as the control, were now left undisturbed in the incubator for 48 hours.) One tenth c c. from each dilution was mixed with 8 c c. melted agar and poured into plates. After 24 hours' incubation the colonies were carefully counted, using a microscope with low power lens. The average number from a standard square in the center and from 2 squares at opposite sides of the plate was used in computing the total number of colonies on the plate. This method of counting was followed in all our subsequent work.

After 48 hours' dilutions, plates and counts were made from the supernatant fluid in the kaolinized culture, and from the control as described above. The results of these two experiments are recorded in table 1.

TABLE 1

EFFECT OF KAOLIN ON GROWTH OF B. COLI

	Colonies on Kaolin Plate	Colonies on Control Plate	Approximate Ratio Between Them
High dilution plate 3-hour culture	28,000	108,000	1 : 4
Low dilution plate 3-hour culture	55,200	149,280	1 : 3
High dilution plate 48-hour culture	64,800	79,200	4 : 5
Low dilution plate 48-hour culture	130,200	149,200	4 : 5

The first experiment indicates that kaolin has no effect on the growth of bacteria in solid mediums. Perhaps an average from a larger number of such plates would have been different.

The second experiment shows that when added to fluid mediums incubated at about body temperature and kept in motion—a condition somewhat analogous to that of a large amount of the substance in the intestine—it carries down with it a large number of bacteria. There were from 3 to 4 times as many colonies on the control plates as on the ones made from the kaolinized culture.

Whether or not there is a definite union between the bacteria and the particles of kaolin, as is the case between it and toxin molecules, is not clear.

Further, when the kaolinized culture as well as the control are left undisturbed for 48 hours, and the plates are inoculated from dilution made from the supernatant fluid, there are almost as many bacteria in 0.1 c c. of this fluid as in an equal amount from the control.

THE EFFECT OF KAOLIN ON BACTERIAL TOXINS AND TOXIC PRODUCTS

To increase their virulence, two animal passages were made of strains of B. typhosus and paratyphosus, B. dysenteriae (Shiga), and B. enteritidis.

They were grown in 500 c c. flasks containing 250 c c. dextrose broth each, for 4 days. They were killed by heating in a water bath at 60 C. for 20 minutes, then filtered through porcelain filters. Five c c. doses of these toxic filtrates were injected into rabbits intravenously. One was injected with 5 c c. dextrose broth as a control.

Five c c. of each toxic filtrate were placed in tubes containing 1 gm. of kaolin each. These were well mixed, incubated for 20 minutes, being shaken every 5 minutes, then centrifuged, and the supernatant fluids injected into rabbits intravenously. In the case of B. botulinus A, 0.5 c c. of a fluid culture, 14 days old, obtained from Dr. Geiger, Chicago University, was used. The exact lethal dose for a rabbit was not determined, but the amount used surely contained several lethal doses. It was diluted with 5 c c. of salt solution and injected subcutaneously. A similar amount of the culture, also diluted with salt solution, was mixed with 4 gm. of kaolin, centrifuged and injected. This experiment was repeated with similar results as recorded in table 2.

TABLE 2

THE EFFECT OF KAOLIN ON BACTERIAL TOXINS AND TOXIC PRODUCTS

Rabbit	Toxin or Toxic Product from	Intravenous Injection of	Result
1	B. typhosus	5 c c. toxic filtrate	Quiet for 24 hours
2	B. paratyphosus B	5 c c. toxic filtrate	Quiet for 28 hours
3	B. enteritidis	5 c c. toxic filtrate	Died after 24 hours
4	B. dysenteriae (Shiga)	5 c c. toxic filtrate	Died after 12 hours
5	B. botulinus A	0.5 c c. fluid culture (subcutaneously)	Died after 10 hours
6	B. typhosus	5 c c. toxic filtrate treated with kaolin	Eating after 6 to 7 hours
7	B. paratyphosus B	5 c c. toxic filtrate treated with kaolin	Eating after 8 hours
8	B. enteritidis	5 c c. toxic filtrate treated with kaolin	Eating after 8 hours
9	B. dysenteriae (Shiga)	5 c c. toxic filtrate treated with kaolin	Eating after 7 hours
10	B. botulinus A	0.5 c c. fluid culture treated with kaolin	Eating after 6 hours
11	(Control)	5 c c. dextrose broth	Eating after 6 to 7 hours

The effect of kaolin on diphtheria toxin was also determined. No. 2a toxin, prepared by Dr. George H. Weaver, was used. The MLD of this toxin was found to be 0.012 c c.. Throughout the experiment the amount taken as one MLD was 0.015 c c., so as to be sure that it would be fatal. In the first experiment single MLD doses were added to 1 c c. of sterile salt solution, shaken up with various amounts of kaolin for 15 to 20 minutes, centrifuged, and the clear, supernatant fluid injected hypodermically into young guinea-pigs weighing about 250 gm.

TABLE 3

THE EFFECT OF KAOLIN ON DIPHTHERIA TOXIN

Guinea-Pig	One c c. Salt Solution Containing 1 MLD Treated with	Result
1	10 mg. kaolin	Died in 88 hours
2	10 mg. kaolin	Died in 66 hours
3	25 mg. kaolin	Lived
4	25 mg. kaolin	Lived
5	50 mg. kaolin	Lived
6	50 mg. kaolin	Lived
7	100 mg. kaolin	Lived
8	100 mg. kaolin	Lived
9	(Control—1 MLD)	Died in 92 hours

The second experiment was made in the same manner as the first, except that a 20% suspension of kaolin was used in place of the powder. The same amount of toxin and kaolin was used as before (0.05 c c. of a 20% suspension = 10 mg. of the powder).

In the second part of this experiment, 5 MLD doses were used, and 5 times as much kaolin-suspension as in the first. Each dose was made up to 2.5 c c. with salt solution (table 4).

Hektoen and Rappaport[22] found that frequent insufflations of kaolin powder into the throats of diphtheria patients, in which large numbers of bacilli were present, removed these completely and apparently permanently in from 2 to 4 days. It was discontinued because of the inconvenience it caused the patients. A thick suspension would probably have been more convenient, and perhaps more effective, as the particles of kaolin would have been more completely separated from one another.

TABLE 4

THE EFFECT OF KAOLIN ON DIPHTHERIA TOXIN, CONTINUED

Guinea-Pig	One MLD + Salt Solution q.s. to Make 1 c c. Treated with	Result
10	0.05 c c. kaolin suspension	Died in 42 hours
11	0.125 c c. kaolin suspension	Lived
12	0.25 c c. kaolin suspension	Lived
13	0.63 c c. kaolin suspension	Lived
14	Control (1 MLD)	Died in 48 hours
	Five MLD + Salt Solution q.s. to Make 2.5 c c. Treated with	
15	0.25 c c. kaolin suspension	Died in 48 hours
16	0.63 c c. kaolin suspension	Lived
17	1.25 c c. kaolin suspension	Lived
18	2.5 c c. kaolin suspension	Lived
19	Control (5 MLD)	Died in 16 hours

The Effect of Kaolin on the Normal Intestinal Flora.—Much time was spent in trying out various mediums and methods in becoming familiar with the types of bacteria normally present in the intestines of rats, dogs, and human beings. Kaolin appeared to decrease the number of gram-negative bacteria in feces and to increase the gram-positive. I then decided to concentrate the attention on its effect on the proteolytic and aciduric groups and on B. welchii.

For this purpose, the 5% acid liver agar medium recommended by Torrey,[23] and that described by Ayers and Rupp,[24] were found most suitable. B. acidophilus does not grow on Ayers-Rupp medium, and gram-negative bacilli, as well as most cocci, do not as a rule grow on highly acid liver agar medium. However, this selective action by the latter is not absolute. A plate inoculated from a suspension of a stool predominantly putrefactive produces a number of colonies of gram-negative bacilli. A plate from the same batch of medium, with the same amount of medium poured into it, and incubated along with

[22] Jour. Am. Med. Assn., 1915, 64, p. 1985.
[23] Jour. of Bacteriol., 1917, 2, p. 435.
[24] Ibid., 1918, 3, p. 433.

the former, but inoculated with a suspension from a predominantly aciduric stool, developed only colonies of gram-positive bacilli, yeasts, and a few cocci. This was observed several times. If as much as 10 c c. of the acid medium were poured into the plate, it would not be as inhibitory as if only 6 or 7 c c. were used. The thinner the plate, the more easily could the morphology of the colony be established clearly.

Technic.—A small amount of stool was put directly into a sterile test tube; about 0.5 gm. was mixed thoroughly with 10 c c. of sterile salt solution. Further dilutions were made from this. Exactly 0.05 c c. of the highest dilution was spread over an Ayers-Rupp plate with a sterile glass rod, and a similar amount over one half of a liver-agar plate; 0.05 c c. of an appropriate dilution of B. acidophilus was spread over the other half for comparison. Another set of plates was inoculated in a similar manner from the next lower dilution. The stool was usually plated within half an hour after it had been passed. The Ayers-Rupp plates were incubated for 24 hours, and the liver-agar plates for 72. The set of plates having a representative number of colonies on them were selected for counting. The number of types of colonies found on the plate was first determined with the aid of the microscope; then one of each stained by Gram's method. Only colonies of gram-negative bacilli were counted on Ayers-Rupp plates, and those of gram-positive bacilli, and which looked like the control colonies on the same plate, were counted on the liver agar plates. Doubtful colonies on the latter were inoculated into dextrose broth, and later into blood agar, litmus milk, gelatine and the sugars for further study.

The advantage of having a pure culture of B. acidophilus as a control on the same liver-agar plate as colonies growing from the stool is obvious. The age of the plate, its thickness, the slight difference in reaction of plates made from different batches of medium, all affect the morphology of the B. acidophilus colonies.

Feeding Experiments on White Rats.—All were young, almost grown-up rats. Fifteen were divided into 3 groups of 5 each. To each of the first group, 5 gm. of ground boiled meat were given per day; to each of the second, 5 gm. of bread moistened in water and sprinkled over with 2 gm. of lactose; to each of the third group, 5 gm. of bread moistened in water and sprinkled with 2 gm. of kaolin. Water was given to all.

It was thought possible that the bread had something to do with the results in those given bread and kaolin. To each of another group of 4 rats 5 gm. of boiled, ground meat mixed with 2 gm. of kaolin were given over a period of 40 days. The results are given in table 6.

Feeding Experiment on Dogs.—Three healthy dogs were used. To each of Nos. 1 and 2, weighing 15 and 18 pounds, 200 gm. of boiled, ground beef mixed

TABLE 5

THE EFFECT OF MEAT, BREAD AND LACTOSE, AND BREAD AND KAOLIN ON THE INTESTINAL FLORA

Rats	Diet	Day of Feeding	Percentage of Aciduric-Proteolytic (A-P) Ratio	Remarks
1	.....	0	4 - 96	Before feeding was begun
6	.....	0	1 - 99	
11	.....	0	1 - 99	
2	5 gm. meat	8	2 - 98	
3	5 gm. meat	12	3 - 97	
4	5 gm. meat	16	1 - 99	
5	5 gm. meat	20	5 - 95	
7	5 gm. bread + 2 gm. lactose	8	85 - 15	
8	5 gm. bread + 2 gm. lactose	12	60 - 40	
9	5 gm. bread + 2 gm. lactose	16	99 - 1	
10	5 gm. bread + 2 gm. lactose	20	92 - 8	
12	5 gm. bread + 2 gm. kaolin	8	97 - 3	
13	5 gm. bread + 2 gm. kaolin	12	80 - 20	
14	5 gm. bread + 2 gm. kaolin	16	99 - 1	
15	5 gm. bread + 2 gm. kaolin	20	92 - 8	
1	5 gm. bread + 2 gm. kaolin	6	92 - 8	Meat group changed to bread and kaolin
3	2 gm. bread + 2 gm. kaolin	10	96 - 4	
12	5 gm. meat	6	50 - 50	Bread and kaolin group changed to meat
15	5 gm. meat	10	1 - 99	
7	5 gm. meat	6	88 - 12	Bread and lactose group changed to meat
8	5 gm. meat	10	80 - 20	
9	5 gm. meat	14	15 - 85	

TABLE 6

EFFECT OF BOILED GROUND MEAT AND KAOLIN ON INTESTINAL FLORA

Rat	Diet	Day of Feeding	Percentage of A-P Ratio
16	5 gm. meat, 2 gm. kaolin	10	95 - 1
17	5 gm. meat, 2 gm. kaolin	16	99 - 1
18	5 gm. meat, 2 gm. kaolin	30	88 - 12
19	5 gm. meat, 2 gm. kaolin	34	98 - 2
16	5 gm. meat, 2 gm. kaolin	35	99 - 1
17	5 gm. meat, 2 gm. kaolin	36	98 - 2
18	5 gm. meat, 2 gm. kaolin	37	95 - 5
19	5 gm. meat, 2 gm. kaolin	38	96 - 4
16	5 gm. meat, 2 gm. kaolin	39	98 - 2
17	5 gm. meat, 2 gm. kaolin	40	95 - 5

with 30 gm. kaolin were given per day. No. 3, that weighed 35 pounds, received 300 gm. of meat and 60 gm. of kaolin.

One c c. from each dilution of 1 : 100, 1 : 500, 1 : 2500 and 1 : 12500 were inoculated into tubes containing about 8 c c. of fresh, whole, sterile milk. The tubes were heated to 80 C. for 20 minutes in a water bath, to kill all vegetative forms, then cooled quickly in cold running water to bring the cream to the surface, thus forming a relatively anaerobic medium for spores of B. welchii to develop in. These were incubated for 72 hours. When there was evidence of the "stormy fermentation" described by Simonds,[25] odor of butyric acid, and large gram-positive bacilli present, the culture was counted positive for B. welchii.

TABLE 7

THE EFFECT OF KAOLIN ON THE INTESTINAL FLORA OF DOGS

Day of Feeding	Percentage of Aciduric-Proteolytic Ratio			Remarks
	Dog 1	Dog 2	Dog 3	
	3 – 97	1 – 99	4 – 96	Before feeding was commenced
5	60 – 40	66 – 44	70 – 30	
8	50 – 50	70 – 30	50 – 50	
11	.....	94 – 6	50 – 50	No. 1 plates spoiled
15	58*– 42	75*– 25	67 – 33	
22	80*– 20	70*– 30	86*– 14	
25	60*– 40	50 – 50	60*– 40	
27	32 – 68	57*– 43	54*– 46	
29	45*– 55	80*– 20	70*– 30	Kaolin stopped
4	50 – 50	70*– 30	50*– 50	
8	20 – 80	25 – 75	90*– 10	

* In these the larger, flat, light brown, and the fuzzy colonies made up of somewhat longer, thinner, gram-positive bacilli, predominated. In the others, the regular edged, smaller, dark brown, heaped-up colonies, just like the control colonies, were most numerous. These types will be described later.

As the flora became predominantly aciduric, spores of B. welchii diminished in number; at times they were absent in all dilutions. They were present in all dilutions before feeding was commenced.

Feeding Experiment in Men.—Three vigorous, healthy men volunteered, together with myself, to take 30 gm. of kaolin per day for one month. Three of us took 60 gm. per day for a while. All continued their usual diet. The kaolin was usually taken in about 2 ounces of water just before retiring, or in the middle of the forenoon.

C and D, while taking 2 ounces a day, had to take a cathartic once or twice, but it was not uncommon for them to have to do so ordinarily. B did not become constipated even while taking that amount. The stools were more copious than usual, and the first part passed was drier than the rest, but there was no diarrhea. Three reported a gain

[25] Monographs of the Rockefeller Institute of Med. Research, 1915, No. 5.

in appetite; two, who weighed themselves before and after the experiment, gained 5 and 3 pounds; two, who had been troubled with flatulency, reported improvement.

In one, the flora remained predominantly aciduric for 8 days after kaolin was stopped and in another for 12 days.

TABLE 8

THE EFFECT OF KAOLIN ON THE INTESTINAL FLORA OF MAN

Day of Feeding	Percentage of Aciduric-Proteolytic Ratio				Remarks
	A	B	C	D	
	2 - 98	1 - 99	1 - 99	8 - 97	Before feeding was commenced. C and D took 60 gm. per day the first 8 days. B took 60 gm. per day from the 8th to 17th day
8	5 - 95	20 - 80	96 - 4	65*- 35	
12	2 - 98	95 - 5	50 - 50	70*- 30	
16	20 - 80	98 - 2	98*- 2	68*- 32	
20	30 - 70	90*- 10	99*- 1	discontinued	
28	55 - 45	89*- 11	98*- 7		
30	61*- 39	95*- 5	40*- 60		
34	50 - 50	90 - 10	50*- 50		
38	10 - 90	5 - 95	60 - 40		
42	.....	.....	55 - 45		
46	.....	.....	15 - 85		

Bold faced figures = date on which kaolin was stopped

TABLE 9

THE EFFECT OF KAOLIN ON THE DEVELOPMENT OF B. WELCHII SPORES IN THE INTESTINE OF MAN

Day of Feeding	B. welchii Spores in 1 c c. of Serial Dilutions Made from Suspensions of the Stools of															
	A				B				C				D			
	1: 100	1: 500	1: 2500	1: 12500	1: 100	1: 500	1: 2500	1: 12500	1: 100	1: 500	1: 2500	1: 12500	1: 100	1: 500	1: 2500	1: 12500
Before feeding	+	+	+	0	+	+	+	+	+	0	+	+	+	+	+	+
8	+	+	0	0	0	+	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16	+	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0
20	0	0	0	0	0	0	0	0	0	0	0	0	discontinued			
26	0	0	0	0	0	0	0	0	0	0	0	0				
30	0	0	0	0	0	0	0	0	0	0	0	0				
34	0	0	0	0	+	0	0	0	0	0	0	0				
38	0	+	0	0	0	0	0	0	0	0	0	0				
42	..	..	..	..	0	0	0	0	0	0	0	0				

+ = culture positive for B. welchii spores.
0 = culture negative for B. welchii spores.
Bold faced figures = kaolin stoped on that date.

Rettger and Cheplin,[26] found that they could change the flora in rats and in man by feeding lactose or dextrin. This has been repeated by Cannon and McNease,[27] and others. Barker,[28] procured favorable results in typhoid patients by feeding them lactose and milk, and

[26] Intestinal Flora, 1920.
[27] Jour. Infect. Dis., 1921, 29, p. 369.
[28] Jour. Am. Med. Assn., 1914, 63, p. 929.

Torrey,[29] observed that feeding a high carbohydrate diet (lactose) to such patients tended to reduce the number of putrefactive bacteria and to increase the aciduric.

In three of the men fed kaolin in this experiment, the flora became predominantly aciduric in 8 to 10 days, in the fourth more slowly. Much smaller amounts of it was taken than is necessary with lactose. It does not cause the patient so much discomfort, nor upset the digestion as lactose frequently does. It also has a protective action on the intestinal mucosa that is of value when this is irritated and damaged by bacteria or their products.

The disappearance of flatulency, when one takes kaolin over a long period of time, is not strange, since the proteolytic bacteria are gradually replaced by aciduric organisms. The fact that the gas bacillus also almost disappears may be a potent factor in the same direction.

Hines,[30] and others have observed that B. welchii spores disappear from the stool when it becomes aciduric.

Klein,[31] Tissier,[32] and Kendall[33] hold that B. welchii are frequently the cause of diarrhea. Shamberg,[34] believes that it is an important factor in poisoning from eating sausages.

Characteristics of Typical Colonies, and Organisms Comprising Them Which Develop from an Aciduric Stool on Acid Liver Agar.—When the flora is beginning to change to one chiefly aciduric, especially in man, the type of B. acidophilus colony found on the liver-agar medium is round, dark brown to light brown (amber) at the edges, coarsely granular (fig. 2), very much like the control colony on the other half of the plate (fig. 1).

Stained by Gram's method, the organism is a small gram-positive bacillus, tapering at the ends, frequently arranged end to end much like a lanceolated diplococcus but having no capsule. Some longer bacilli are also seen. Two or three lying parallel is characteristic (fig. 4). They look very much like those from the control colonies (fig. 3).

When the flora has remained aciduric for some days, another type of colony appears in increasing numbers; one that is larger than the former, flatter, a lighter brown, not so round and with less regular

[29] Jour. Infect. Dis., 1915, 16, p. 72.
[30] Jour. Infect. Dis., 1923, 32, p. 280.
[31] Report of Med. Officer to Loc. Govt., 1896, Vol. 25 supplement, p. 173.
[32] Ann. de l'Inst. Pasteur, 1905, 19, p. 273.
[33] Jour. Med. Res., 1911, 25, p. 117.
[34] Ztschr. f. Hyg. u. Infektionskrankh., 1902, 41, p. 183.

edges (fig. 5, b). These are also composed of gram-positive bacilli much like the control, but a trifle thinner (fig. 6). A third type of colony is also found, especially in specimens from dogs, which is fuzzy, dark brown at the center, lighter toward the edges; at times the whole colony is quite light. It has numerous strandlike outgrowths at the edges making it appear much like a bunch of cotton (fig. 5, c). It is composed of gram-positive bacilli which in the original colonies (direct from the stool) are thinner and longer than the first two described; they grow in long threads lying parallel. This arrangement disappears soon when transferred to other mediums. Fewer long forms, too, are then found (fig. 7).

As the flora begins to revert back to the proteolytic type, the B and C types of colonies disappear first, leaving sometimes almost a pure culture of type A colony in the liver agar medium. This is most pronounced in stools from man.

The question arises: Do these different-looking colonies represent different types of a group of aciduric bacilli whose biologic reaction in various culture mediums are much alike, or are they different manifestations of a pleomorphic organism which appears in different shapes, depending on whether its environment is favorable or not?

It was noticed that the larger, light brown colonies, as well as the fuzzy ones, lost much of their original appearance after having been transferred into dextrose broth, incubated for 24 hours, and again seeded on acid liver agar. This process was therefore repeated 5 times. It was then found that type *B* colony was producing a large number of colnies similar to type *A* and the control colonies (fig. 8). Likewise type *C* colony had lost its fuzziness, and was now producing colonies which for the most part resembled the large light brown colony (type *B*); a few round, dark brown colonies were also seen, much like the control colonies (fig. 9). In stained preparations, the bacilli of both types were now more like the control than before.

Subcultures of the three types of colonies and of the control were carried through dextrose broth, litmus milk, gelatine, blood agar and the sugars. In dextrose broth, after 24 hours' incubation, the growth from types *B* and *C* were more scanty than that from type *A* and the control. After making subcultures a few times, it became more abundant. In all types the broth tubes remained quite clear, except near the bottom where they were a trifle cloudy, and the bacteria seemed to adhere to the sides of the tubes. On the bottom of all there was a white sediment of bacteria. All turned litmus milk acid. After from

3 to 4 days, a white coagulum began to form at the bottom of the tubes, extending gradually upward, without separation of serum. This was repeated a number of times. The coagulum did not commence forming at the same time in all tubes, but it eventually appeared. The growth of all types in gelatine was uncertain. After 5 days' incubation an almost imperceptible growth of each was obtained on blood agar; there was no hemolysis and no green discoloration of the medium. After 48 hours' incubation, all types had fermented dextrose, maltose and saccharose. After 72 hours, lactose and mannite would usually be slightly acid. Salicin, dulcite and inulin were never fermented. No gas appeared in any of the tubes.

Streptococcus fecalis, with which the bacillus from type *A* colony might be confused, makes dextrose broth uniformly cloudy; it grows well on blood agar; it ferments salicin, and in dextrose broth it develops long chains. Its colonies are finely granular and of a dirty grayish brown at the center to gray at the edges, in contrast to the amber brown edges of colonies of this bacillus growing on acid liver agar.

There is a possibility that the 3 types of colonies, *A*, *B*, and *C*, represent different forms of growth of the same organism. When the pabulum in the intestine begins to become favorable for the growth of aciduric organisms, the B. acidophilus forms colonies such as type *A*, just like those produced by it when it has grown on artificial mediums for a long time. When the environment becomes more favorable for its development, it produces colonies of the *B* and *C* type as well.

The other possibility is that these three types of colonies represent different organisms belonging to the same group, which culturally behave very much alike. If this is so, better results should be obtained when B. acidophilus is fed to patients if a mixed culture is used.

CLINICAL OBSERVATIONS

Asiatic Cholera.—A study of 100 consecutive cases was made during the epidemic referred to, and reported to the Conference of the China Medical Missionary Association, Peking, in February, 1920 (Braafladt [35]).

Microscopic preparations stained with one tenth strength carbol fuchsin were made from small, white flakes taken from the thin, watery stools of a large number of cases. These contained almost a pure culture of gram-negative bacilli. Some were slightly bent,

[35] China Med. Jour., 1920, 34, p. 243.

tapering at the end much like cholera vibrio. Judging from the smears, it was not possible to be sure that the organisms were not colon bacilli, although they were very small. When grown on artificial mediums, they could not be thus mistaken. For lack of time, not many strains were studied. One was observed over a long period of time. In the original smear this strain did not seem different from the others. The patient from whom it was taken recovered.

One c c. of a 24-hour broth culture of this strain caused the death of a guinea-pig in 24 hours, injected intraperitoneally. The vibrio was recovered in pure culture from the serous peritoneal fluid at necropsy. A slant agar culture, one week old, contained numerous involution forms; it liquefied gelatine; growth was much augmented by incubating for 24 hours in salt peptone solution; it agglutinated very well with serum from convalescing patients.

There is probably no other disease amenable to treatment the death rate of which can be reduced so markedly by beginning treatment early as cholera. This seems especially true when kaolin is used. Of the 17 deaths in this series, only 1 occurred among the 42 patients that entered on the first day of illness; 4 of the 24 that entered on the second day died, and 12 of the 34 that entered on the third day or later.

It seems that the copious watery evacuations and vomiting are due primarily to the local irritative action on the intestinal mucosa of the toxins liberated by the rapidly multiplying bacteria. Those symptoms which come on after the diarrhea and vomiting have persisted for some time, such as cramps, hoarseness, failing vision and deafness—the syndrome of collapse—may be considered as secondary and, in part at least, due to the effect of the toxins entering the blood stream. If this interpretation is correct, it is not difficult to understand why kaolin taken in large quantities early in the disease checks it so effectually.

To facilitate study, the patients were placed in two groups according to the severity of the symptoms at the time of admission. Those severely ill were placed in group A, those moderately ill in group B; there were 67 in the first group and 33 in the second. Table 10 shows the relative frequency of the symptoms observed in this series, and the number of patients that died in each group.

As soon as the patient was admitted the pulse rate, respiratory rate, and the axillary and rectal temperatures were recorded. The low axillary temperature and elevated rectal temperature recorded by

Manson,[36] was not commonly observed by us. The greatest difference recorded was 5 F. If we had taken the temperatures of the fatal cases shortly before death, the difference would undoubtedly have been greater.

White cell counts were taken in only 5 cases. All had a leukocytosis. The increase in number of white blood cells is undoubtedly a relative one, owing to the marked anhydremia. MacCallum[37] states that the red cells are far above 5,000,000 per c. mm. The increase in specific gravity of the blood is most likely due to the same cause. The frequency of oliguria and the development of toxic symptoms induced us to try the phenolsulphonephthalein renal function test in a few patients, one of whom had developed toxic symptoms. All the 10 patients tested had sustained injuries of the kidneys. The tardy appearance

TABLE 10

GROUPING OF PATIENTS BASED ON SYMPTOMS PRESENT ON ADMISSION TO THE HOSPITAL

	Of the Total 100 Cases	Group A (67)		Group B (33)	
		Number	Percentage	Number	Percentage
Copious watery stools	100	67	100	33	100
Vomiting	100	67	100	33	100
Cramps	60	58	86	8	24
Hoarseness	54	48	72	6	18
Deafness	19	18	27	1	3
Failing vision	6	6	9	0	0
Died	17	15	22	2	6

of the drug in the urine, and the small amount excreted during the first hour as compared with the large amount excreted during the second hour indicated that the renal cells had not lost their power of excreting, but that the process was slowed up due to the swelling of the cells and the lowered blood pressure.

Treatment.—The wards on the ground floor were given over to cholera patients; ordinary patients were cared for on the second and third floors. Cheap Chinese beds covered with straw matting and hospital bedding consisting of a sheet and one or two blankets were used. The straw mats were usually burned when the patient left the hospital; the bedding was soaked in 5% phenol for some hours and dried in the sun before being given to the next patient. The floors were mopped with antiseptics once or more a day. The food for the cholera patients came from the general kitchen, but all dishes were boiled before returning. Almost all the physicians, interns, nurses

[36] Tropical Diseases, Ed. 5, p. 462.
[37] Text Book of Pathology, 1918, p. 449.

and coolies working in the cholera wards were vaccinated against cholera. None of these contracted the disease, nor did a single case develop among the ordinary patients on the floors above.

On admission the patients received 1 c c. of brandy and ether, half and half, subcutaneously; if restless, an eighth or a sixth of a grain of morphine. If the radial pulse was very weak or could no longer be felt and there was danger the patient would die in a short time, he was given hypertonic salt solution intravenously at once. It was introduced by gravitation at an average rate of 1 liter per half hour. The formula used was that recommended by Rogers: sodium chloride 120 grains, potassium chloride 6 grains, calcium chloride 4 grains, to one pint of water; then sterilized. From 1 to 3 liters were introduced at a time. Frequently after some hours a second injection was necessary, and sometimes a third.

In the beginning all patients were given salt solution. Some, however, who were not in severe collapse became very uncomfortable after the injection.

Having read an abstract of an article written by Kuhne,[38] wherein he reports that he had given up all other forms of treatment, including hypertonic salt solution, for kaolin, reducing his mortality from 60 to 3% among soldiers suffering with cholera during the Balkan war in 1910, we decided to try it for those moderately ill. Since it proved satisfactory, we later used it also for those severely ill. Table 11 shows that 15 in Group A (severely ill) received this treatment, as compared with 20 in group B (moderately ill). It was given by mouth in a heavy suspension, about as thick as a thin gruel: 800 gm. stirred up in 1,000 c c. of water. The vomiting ceased more quickly when a thick suspension was used than when a thin one was used. If the vomiting was severe, 3 ounces of the suspension were given every half hour. After from 6 to 8 hours, the vomiting abated and the diarrhea decreased. It was then given only once every hour, and finally every 2 hours. As a rule, it was not necessary to give kaolin over a period of more than 12 to 15 hours, and frequently 6 to 7 hours sufficed. No food was given during the time kaolin was being administered.

Only one of the 35 patients who received kaolin died; this patient was unconscious at the time of admission, and had complete suppression of the urine, so that it is a quesion whether any form of treatment would have helped her.

[38] Rev. Suisse Romande, 1918, 38, p. 555.

The average number of days spent in the hospital by each patient, the total number of patients receiving salt solution treatment only, salt solution and kaolin, or kaolin only, as well as the number that died in each of these groups are given in table 11.

In the autumn of 1920, three middle-aged men suffering with cholera were admitted to the hospital. All were in collapse when admited, the radial pulse could no longer be felt at the wrist in two of them, and it was very weak in the third. Two of them were given kaolin, one hypertonic salt solution. The two given kaolin recovered and the third, who received salt solution, died.

Wylie [39] reported good results with kaolin during the 1919 epidemic at Paotingfu, but gives no details. Walker [40] used it at Foochow and

TABLE 11

THE RELATION OF THE VARIOUS FORMS OF TREATMENT TO THE LENGTH OF STAY IN THE HOSPITAL AND TO THE DEATH RATE

Treatment Received	Of the Total 100 Patients	Average Number of Days in Hospital	Number that Died
Salt solution only	41	8	9
Salt solution plus kaolin	24	6	7
Kaolin only	35	4	1

TABLE 12

AVERAGE AMOUNT OF HYPERTONIC SALT SOLUTION, KAOLIN, OR BOTH GIVEN TO PATIENTS

	Hypertonic Salt Solution, Cc.	Salt Solution, and Kaolin, Cc.	Gm.	Kaolin, Gm.
To those that recovered	4,130	4,300	800	1,078
To those that died	5,050	3,570	913	2,150 (1 case)

Hinghua the same summer, in 75 cases at the latter place, without any deaths. There is a possibility that some of these were not genuine cholera cases, as the diagnosis was based only on clinical manifestations. Some of the patients were in extreme collapse when treatment was begun. Crawford [41] used kaolin during the epidemic of 1921 at Tzeluitsing, and had a mortality of 20%. He says: "Undoubtedly kaolin is of great value, but it is an absolute failure if not taken EARLY in the disease." The explanation of his rather high death rate, as well as of his conclusion, probably lies in the fact which he records earlier in his communication, that he telegraphed for kaolin but

[39] China Med. Jour., 1920, 34, p. 252.
[40] Proc. Royal Med. Soc., 1921, 14, p. 23.
[41] China Med. Jour., 1921, 35, p. 417.

did not receive it until after the epidemic was over. In the meantime, he secured some earth locally from the Luchow porcelain works, and used it on the cases he reports. The uncertainty of the adsorptive power of impure and unstandardized clays may create impressions that are erroneous. It is a question just how much value to place on results obtained with them.

When my series of cases was reported at the Conference in Peking, February, 1920, I made the following comment:

A great deal of work on the chemistry of colloids has been done during the last few years, but the field is as yet largely unexplored. It has been fairly well established that there is an adsorptive interaction between colloids and crystalloids, and according to Young,[42] it has been found that a similar interaction takes place between two opposite electrically charged colloids when in proper concentration.

It is highly probable that when kaolin is taken in large quantities by mouth by a patient suffering with cholera, it quickly combines with the toxins liberated in the intestine rendering them harmless. It is also probable that it makes a rather unfavorable medium for the bacteria to multiply in. Those of our patients who received kaolin only made a much quicker recovery than the others. None of them developed the distressing and dangerous toxic symptoms which many of the others did.

In 1921, while at home on furlough in England, Dr. Walker showed experimentally that, so far as the effect of kaolin on cholera vibrio and its toxins is concerned, this interpretation, based largely on clinical evidence, was essentially correct. Some of his results are:

(1) In a rabbit fed 20 c c. of a 50% kaolin suspension, and a similar amount given per rectum, when chloroformed 42 hours later, traces of kaolin were found adhering to the wall of the small intestine high up, and this increased in amount to the cecum, while the colon was filled with kaolin and feces.

(2) Filtrates from alkaline peptone cultures—the organism being passed through 3 to 5 guinea-pigs first to increase its virulence—as well as filtrates from the killed, ground-up and washed vibrios, killed rabbits when injected intravenously in 2 c c. doses. These toxic filtrates lost their toxicity when shaken up with kaolin.

(3) Growths on subculture were obtained in all cases in which different amounts of kaolin had been added to broth cultures of the vibrios, showing that it had no bactericidal effect.

He concludes that the effect of kaolin on the vibrio in the intestine is a purely mechanical one, and on the toxin liberated by them, one of adsorption.

Dysentery.—CASE 1.—A child, 5 years old, had been suffering for 7 days from tenesmus and frequent loose bowel movements which contained mucus and

[42] Zinsser: Infection and Resistance, 1919, p. 556.

blood. He was anemic, weak, fretful, and did not care for food. No amebas were found, and 3 injections of emetine had no effect. Shiga's dysentery bacillus, common in north China, did not grow. Clinically, the condition was like bacillary dysentery and it was treated as such, but with poor results. On the 11th day, the child was still having 20 to 30 stools in 24 hours. A suspension of 3 ounces of kaolin in water and simple syrup was given in 5 doses, one each hour. The following day he had 3 bowel movements, which were formed. He was kept on a light diet for some days, and recovery was complete.

CASE 2.—A child, 14 months old, developed small, loose, greenish bowel movements about every half hour. They contained blood and mucus. The temperature ranged from 100 F. to 104 F. He was fretful and losing strength rapidly. No amebas were found, but a growth of Shiga's dysentery bacillus was obtained. The usual forms of treatment were of no avail. On the 7th day, he was given 2 ounces of kaolin in water and syrup. The following day, he had 3 bowel movements and on the next, 2; these were formed. He had no more trouble.

CASE 3.—There was mucus in the stools of a man who had repeated attacks of "dyspepsia" every summer for a number of years. Several examinations failed to reveal amebas or dysentery bacilli. He had 5 to 8 bowel movements per day, and a dull pain in the lower part of the abdomen. He was not given any food for 24 hours, and treated with bismuth and salol. Only milk was given the second day. On the third day he was in pain as before, and the diarrhea continued. He was then given 300 gm. of kaolin in water, divided into 5 hourly doses. The pain disappeared the next day and the stools became formed. On the fourth day after the kaolin had been taken, the pain and diarrhea reappeared. The stool was again examined, and a few amebas were found. After a thorough course of oil of chenopodium and emetine the condition cleared up.

In the first two cases of bacillary dysentery, the beneficial action of kaolin can be accounted for by the adsorptive effect it has on the toxin liberated by this organism, as well as by its protection of the inflamed mucosa. In the case of chronic amebic dysentery, we see that its effect was only temporary, owing largely to its protective action on the mucosa.

Chronic Ulcerative Colitis.—An elderly man who had had several attacks of amebic dysentery for which he had been treated, complained of recurring dull pain in the lower epigastrium and inability to eat anything but light food. The stools contained mucus, slight traces of blood, and pus. Proctoscopic examination revealed small shallow ulcers on an inflamed and thickened mucosa. No amebas or cysts were found. He was given one ounce of kaolin in water per day for a month. The pain disappeared after the second or third day, the bowel movements were reduced to 2 or 3 per day. The pus, blood, and most of the mucus disappeared from the stools. He was able to eat a more varied diet than before. As he was lost from observation, it is not known whether improvement was permanent.

Here, too, the protective action of the kaolin on the inflamed and ulcerated mucosa is probably its most important action, but the tendency it has to change the intestinal flora from a proteolytic to an aciduric type when taken over a long period of time may also have a beneficial effect in these cases. It may also adsorb some of the toxic products resulting from the extensive putrefaction which takes place in a bowel that is ulcerated. (This will be discussed later.)

Acute and Chronic Enteritis.—Through the permission of Dr. W. J. Quigly, attending physician at Cook County Hospital, and with the co-operation of Dr. L. E. Hines, three patients with pulmonary tuberculosis, one with acute enteritis and two with chronic enteritis, were treated with kaolin over a period ranging from 1 to 6 weeks. In all, the pain disappeared after a few days, so did most of the mucus, and the number of bowel movements were reduced, while the appetite increased. One gained 5 pounds in 2 weeks, and one 21 pounds in 6 weeks. In the last named, the flora became predominantly aciduric. Gas bacillus spores, too, diminished in number in the stools.

Hess [43] used kaolin in infant diarrhea without much success. He does not state the amount given. He says only that it was given by the "teaspoon." He probably did not give enough to obtain results.

DISCUSSION

That kaolin taken over a long time tends to change the intestinal flora to an aciduric type indicates that such a change does not depend wholly on the presence in the intestine of utilizable carbohydrate (lactose or dextrine). Meat, kaolin, and water fed to rats and dogs over a period of from 10 to 30 days produces a similar effect. Just how kaolin produces this effect is difficult to say; it may be by absorbing the toxic products of proteolysis, thus making the pabulum in the intestine unfavorable for the development of proteolytic bacteria.

Dragstedt [44] found that thyroparathyroidectomized dogs can be kept alive for at least a year by feeding large amounts of lactose, thus changing the intestinal flora and ridding the intestine of the toxic products of proteolysis. He believes that the tetany, or depression, from which those dogs that have not been fed lactose die in a few days is due to the absorption of these products.

[43] Jour. Am. Med. Assn., 1916, 66, p. 106.
[44] Am. Jour. Physiol., 1923, 64, p. 424.

He consented to try kaolin instead of lactose on some of the dogs, and he has given me permission to state that in preliminary experiments the results are that kaolin in 100 gm. doses per day prevents the onset of tetany in much the same manner as does lactose.

This points to an adsorption of toxic products of proteolysis by kaolin just as it adsorbs the toxins and toxic products of pathogenic bacteria multiplying in the intestine. Or, it may be due largely to the fact that it changes the type of bacteria in the intestine, and that the toxic effect of the products of proteolysis is for that reason diminished —the proteolytic bacteria being practically eliminated.

The exact nature of the reaction that takes place between kaolin and toxins, or toxic products, is not fully understood, but it is probably an electrical one. Bayliss,[45] Macleod[46] and others hold that kaolin carries an electronegative charge. This may change to a positive charge when in an acid suspension.

The prompt neutralizing effect that kaolin has on toxins and toxic products of pathogenic intestinal organisms when these are treated with it, centrifuged, and the supernatant fluid injected into animals, largely explains the favorable results obtained with it in the patients suffering with Asiatic cholera, bacillary dysentery, and acute enteritis reported in this paper. It would also seem to indicate that it might be beneficial in typhoid, meat poisoning due to the enteriditis-parathyphoid group, and in botulism, especially if given early in the disease.

SUMMARY AND CONCLUSIONS

Kaolin does not act as an antiseptic, but it "carries down with it" large numbers of bacteria in fluid mediums when mixed with them and kept in motion for 2 to 3 hours. Whether this is a purely mechanical action, or whether kaolin has some specific attraction for bacteria, is not clear. When not kept in motion it does not reduce the number of bacteria to any extent.

It combines with toxins and toxic products of Vib. cholerae, B. dysenteriae (Shiga), B. enteritidis, B. diphtheriae, B. botulinus, B. typhosus, B. paratyphosus B., and, perhaps, of putrefactive and proteolytic bacteria, rendering them harmless.

When taken by mouth over a period of from 10 to 30 days in sufficient quantities (1 to 2 ounces per day for a man), it changes the

[45] Quoted by Walker, Proc. Royal med. Soc., 1921, 14, p. 23.
[46] Physiol. and Biochem. in Modern Medicine, 1918, p. 55.

intestinal flora of rats, dogs and men from a predominantly proteolytic type to an aciduric one.

It practically eliminates B. welchii from the stool; it does not upset digestion.

It has been used with success in Asiatic cholera, bacillary dysentery, chronic ulcerative colitis and acute enteritis.

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Vitamin A Deficiency in Chicks Produced by Adding High Levels of Bentonite to Synthetic Diets

G. M. Briggs and M. R. Spivey Fox

Laboratory of Biochemistry and Nutrition, National Institutes of Health, Public Health Service, U. S Department of Health, Education, and Welfare, Bethesda, Maryland

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BENTONITE is a naturally occurring clay of volcanic origin with many industrial uses. It has extremely small particle size and unusual adsorptive properties. Several types of bentonite exist composed of a variety of minerals (chiefly, montmorillonite) and containing principally aluminum silicate with lesser amounts of oxides and silicates of iron, magnesium, calcium, sodium, with a variety of minor elements. "Sodium bentonites" contain up to 3 percent of sodium, whereas "calcium bentonites" contain 1 or 2 percent of calcium and little sodium.

Sodium bentonite has been used for many years in crate-fattening mashes for poultry and in recent years it has been widely used by the feed manufacturing industry as a "lubricating" agent and binder in pelleting processes. Levels from 1 to 2.5 percent are commonly used in pelleted poultry feeds.

Our interest in bentonite grew out of various published and unpublished observations of possible growth stimulating activity of soil or earth materials in practical feeds for various animals. For example, Kelly, Dearstyne and Lucas (1952) reported that feeding 7 percent of air dried topsoil to turkeys receiving practical diets (composition not given) reduced the incidence of swollen hocks from 85 to 29 percent at 28 weeks of age. A total of 100 turkeys was used per lot. Although full details have not been published, work performed at the Minnesota Agricultural Experiment Station indicated that the feeding of 5 or 10 percent of soil or sand to battery fed chicks receiving practical diets may improve growth at 4 weeks of age (Briggs, 1949). Kratzer and Grau (1954) stated that feeding 2.5 percent of "natural earth materials" (of undisclosed nature) did not improve the growth of young chicks. In preliminary studies with lambs, Jordan (1953) reported that the feeding of sodium bentonite resulted in improved gains and feed consumption. However, additional experiments produced inconsistent results (Jordan, 1954).

In view of the above experiments, we added bentonite to a synthetic diet for chickens. Rather than improved growth, the bentonite caused poor growth, mortality, and symptoms of vitamin A deficiency.

The purpose of the experiments reported here was to determine the effect of bentonite in synthetic diets for the chick and to find the effect of particle size of bentonite on production of vitamin A deficiency. A preliminary abstract of this work has appeared (Briggs and Spivey, 1954).

Two other studies on detrimental effects of bentonite in animals fed synthetic diets were published after our studies were in progress. Laughland and Phillips (1954a, b) in studies with rats found that the feeding of low levels of sodium bentonite (0.5 to 3.5 percent) in synthetic diets caused a lowering of the stores of vitamin A in the liver and changes in liver size. No marked effects on growth or changes in mortality rates were seen in their short time experiments. Also, Wilson

(1953a, b, 1954) found that mice fed a diet containing 50 percent of bentonite failed to grow normally and developed a deficiency of choline and possibly other factors.

EXPERIMENTAL

Day-old sexed New Hampshire chicks from a commercial hatchery were used throughout. They were kept in electrically heated batteries with wire mesh floors. Feed and water were supplied *ad libitum* and feed consumption records were kept. All experiments were conducted for 4-week periods and the chicks were weighed weekly.

Details of the synthetic diet (C2) used have been published previously (Fox, Ortiz and Briggs, 1955). In general, the diet was composed, in percentages, of glucose (Cerelose) 61.5, vitamin-free casein 20, gelatin 8, methionine 0.3, corn oil 4, chick salts A 6, and adequate levels of all known vitamins including 3 mg. (8,720 I.U.) of synthetic vitamin A acetate (about 3 times the requirement suggested by the National Research Council) and 10 mg. of dl-alpha-tocopherol acetate per kg. of diet. The vitamin A acetate and dl-alpha-tocopherol acetate were added to the diet by dissolving the pure materials in a portion of the corn oil. Diets were made fresh for each series and refrigerated, except for portions kept in the animal room for feeding purposes for periods not exceeding 10 days.

The various types of bentonite used were samples of commercially available materials and were kindly supplied by the International Minerals and Chemical Co., Chicago, Illinois, and the American Colloid Co., Chicago, Illinois. All results were obtained with sodium bentonite (200 mesh) unless otherwise indicated. Additions to the diet were made at the expense of glucose.

Vitamin A in the diets was determined by extracting the unadsorbed vitamins with hexane and measuring the color produced by the Carr-Price antimony trichloride reaction.

RESULTS

Table 1 represents a summary of 10 experiments in which 5 percent of bentonite was added to the synthetic diet C2. Bentonite feeding resulted in poor weight gains, in the production of gross signs of vitamin A deficiency in just 2 to 3 weeks, and in high mortality by 4 weeks in spite of the presence of vitamin A in the diet.

The results shown in Table 2 demonstrate the activity of additional vitamin A in overcoming the detrimental effect of bentonite. Good growth and prevention of symptoms were obtained by supplementation with various amounts of vitamin A acetate (fed or injected), beta carotene, or stabilized vitamin A (groups 4 and 8 to 11). The minimum effective level of vitamin A in overcoming the bentonite action was not determined. Neither high levels of the B vitamins (except choline) nor alpha-tocopherol acetate were active in overcoming the deficiency (groups 5 and 12). From the limited data it appeared that the presence of fat in the diet (Crisco or corn oil) was necessary for the destructive action of bentonite since in the absence of fat no symptoms of vitamin A deficiency were seen (compare group 13 with 1 and 14).

TABLE 1.—*Effect of bentonite on mortality and weight of chicks fed synthetic diets*
(Summary of 10 experiments with mixed sexes)

Supplement to diet C2	Number started	Number of survivors at 4 weeks	Gm. weight of survivors at 4 weeks
None	60	60	347
5% bentonite	60	15	165

TABLE 2.—*Effect of various supplements on bentonite toxicity*
(6 chicks started per group)

Group	Supplement to diet C2	No. survivors at 4 weeks	Gm. wt. of survivors at 4 weeks and standard error*	Feed efficiency†
	Series 63—females			
1	None	6	348 ± 12	0.590
2	Minus vitamin A	1	181	.474
3	5% bentonite	1	190	.484
4	5% bentonite+5 mg. β-carotene/kg.	6	300 ± 18	.532
5	5% bentonite+10×all B vitamins except choline	1	190	.481
	Series 67—females			
6	None	6	380 ± 26	.592
7	5% bentonite	2	182 ± 12	.364
8	5% bentonite+27 mg. vitamin A acetate/kg.	6	349 ± 24	.570
9	5% bentonite+vitamin A acetate injected 3× weekly subcutaneously (equivalent to that consumed by preceding group)‡	6	370 ± 13	.596
10	5% bentonite+45 mg. β-carotene/kg.	5	362 ± 34	.576
11	5% bentonite+3 mg. "stabilized" vitamin A/kg.§	6	348 ± 12	.565
12	5% bentonite+90 mg. vitamin E/kg.	0	(all dead)	—
13	5% bentonite minus corn oil	6	338 ± 30	.533
14	5% bentonite minus corn oil+3% Crisco	3	230 ± 19	.433

* Standard error calculated by method of Mantel (1951).

† Feed efficiency = $\frac{\text{total weight gained (of all animals)}}{\text{total feed consumed}}$.

‡ Vitamin A acetate in 50 percent alcoholic solution for the first 2 weeks and in corn oil from 2 to 4 weeks.
§ Supplied in the form of NOP-DRY, Nopco Chemical Co., Harrison, N. J.

The degree of the deficiency was dependent upon the level of bentonite used in the diet (see Table 3). Consistent (but statistically non-significant) growth depressions were obtained in 3 experiments with only 1 percent of bentonite in the diet, although there were no signs of vitamin A deficiency with this level. The chicks receiving 2 percent of bentonite showed the characteristic eye symptoms of vitamin A deficiency and growth was slightly depressed (although not significantly). Pronounced signs of deficiency, including urates in the ureters, poor growth, eye symptoms, and high mortality, were produced with 3 to 10 percent of bentonite.

TABLE 3.—*Effect of various levels of bentonite*
(6 chicks per group)

Level of bentonite added to diet C2	Gm. wt. at 4 weeks, standard error, and survivors*		
	Series 60 (females)	Series 69 (females)	Series 71 (males)
0	354 ± 8 (6)	361 ± 20 (6)	376 ± 22 (6)
1%	324 ± 22 (6)	347 ± 6 (6)	365 ± 21 (6)
2%	—	—	317 ± 26 (6)
3%	—	205 ± 10 (3)	194 ± 14 (2)
5%	133 ± 21 (2)	115 ± 4 (2)	—
10%	—	(all dead)	—

* Number of survivors indicated in parenthesis.

Table 4 shows the effect of particle size and type of bentonite on the production of vitamin A deficiency symptoms. All 4 samples of sodium or calcium bentonites which passed through a number 200 sieve produced vitamin A deficiency, whereas the several samples of bentonite tested of sieve number 90 or larger size were not detrimental at the 5 percent level. The fact that 20 percent of bentonite (sample E) of relatively large particle size (90

TABLE 4.—*Effect of particle size of bentonite and other adsorbing agents on growth and mortality of chicks*
(6 chicks started per group)

Supplement to diet C2	Sieve number*	Sample†	Number of survivors at 4 weeks	Gm. wt. of survivors at 4 weeks and standard error	Vitamin A deficiency signs
None	—	—	6	344 ± 19	—
5% Na bentonite	200	A	1	135	+
5% Na bentonite	200	B	1	250	+
5% Ca bentonite	200	C	1	133	+
5% Na bentonite	200	D	3	168 ± 20	+
5% Na bentonite	90	E	6	332 ± 25	−
20% Na bentonite	90	E	0	(all dead)	+
5% Na bentonite	80	F	6	326 ± 22	−
5% Na bentonite	50	G	6	384 ± 16	−
5% kaolin	200	H	5	346 ± 24	−
5% Norite	200	I	0	(all dead)	?

* The sieve number gives the number of openings per linear inch.
† Sample A was used in all previous experiments. The other samples were commercial bentonite. Samples D, E, and G were the same bentonite but of different grind.

mesh) produced vitamin A deficiency shows that the differences depend on the level of bentonite fed as well as particle size.

Kaolin, also a naturally occurring clay, did not cause signs of vitamin A deficiency at a level of 5 percent. However, the feeding of Norite, a potent adsorbent, resulted in high mortality. No attempt was made to overcome the detrimental effect of Norite by feeding vitamin A and, since the deficiency signs were not specific for vitamin A, it is probable that other vitamins were involved.

TABLE 5.—*Studies on the effect of 5 percent of bentonite on vitamin A levels in diet C2*

Diet	% of original vitamin A (3.0 mg./kg.) remaining in diet		
	2 hrs. after preparation	Stored 5 days at 5°C.	Stored 10 days at 26°C.
C2	100	96.1	89.2
C2+5% Na bentonite (200 mesh)	62.9	41.5	0

It was of interest to know if bentonite destroyed vitamin A in the diet before or after actual feeding to the chick. In an attempt to answer this question the vitamin A content of the synthetic diet was determined at various intervals after mixing. The results, presented in Table 5, show that a marked decrease in the vitamin A content occurred in the bentonite diet within a short period of time, whether

TABLE 6.—*Inactivity of bentonite in causing vitamin A deficiency when added to a practical chick diet (male chicks)*

Supplement to practical diet*	Number of chicks	Number of survivors	Gm. wt. at 4 weeks	Vitamin A deficiency signs	Feed efficiency
None	18	18	389	none	0.478
5% Na bentonite†	18	18	383	none	.472
10% Na bentonite†	6	6	365	none	.403
20% Na bentonite†	6	6	293	none	.302

* The diet used was "Startena" mash manufactured by the Ralston-Purina Company.
† 200 mesh.

refrigerated or not. The decrease would be sufficient to explain the vitamin A deficiency signs produced by feeding the unrefrigerated diet.

Because of the widespread use of bentonite in practical feeds, several experiments were conducted in which bentonite was added to a practical chick diet containing vitamin A activity from natural sources. The results are summarized in Table 6. No signs of vitamin A deficiency were produced by feeding up to 20 percent of bentonite under these conditions. However, 20 percent of bentonite caused a significant depression in growth and feed efficiency which may have been due to a "dilution effect" or to adsorption of other nutrients by bentonite.

DISCUSSION

It is evident from the results that vitamin A deficiency in chicks may occur as a result of adding 2 to 5 percent of bentonite (finely powdered) to a synthetic chick diet. The results secured depend not only on the particle size of the bentonite, but upon the type of diet and vitamin A used. Thus, adding up to 20 percent of bentonite to a practical diet or 5 percent of bentonite of large particle size (90 mesh or larger) to a synthetic diet did not cause vitamin A deficiency signs. All signs of deficiency were prevented by the use of stabilized vitamin A in the diet or by feeding or injecting large amounts of vitamin A.

The results are not entirely unexpected in the light of previously published studies on the destructive action of adsorbing agents (including bentonite) on vitamin A (see references cited by Laughland and Phillips, 1954b). Also, Vecher (1948) reported that in chromatographic studies bentonite adsorbed vitamin A and carotene from certain solvents with the development of specific colors. This property was used by Vecher to separate quantitatively vitamin A from natural carriers. Also, the results showing the destructive action of 5 percent of Norite were not unexpected since Almquist and Zander (1940) demonstrated that the feeding of charcoal caused signs of deficiency of several vitamins in chick diets, including vitamin A. Matet and Matet (1945) found that 2 percent of charcoal in rat diets produced a vitamin A deficiency.

Our results are in general agreement with the conclusions of Laughland and Phillips[1] (1945a, b) who studied the effect of bentonite in rat diets. However, certain important differences exist between the two studies. In our studies, for instance, protection against the deficiency caused by feeding bentonite was obtained by feeding carotene and a stabilized vitamin A preparation in the diet. Also, the two studies differ in the method of measuring the vitamin A status of the animal as affected by bentonite in the diet. In the studies presented here growth and production of gross signs of vitamin A deficiency were studied, whereas Laughland and Phillips measured liver storage of vitamin A. In addition, our studies

[1] Laughland and Phillips reported that in the presence of stabilized vitamin A acetate (20,000 I.U. per kg. of diet) the feeding of bentonite resulted in reduced liver storage of vitamin A and in depressed growth of depleted rats in 6 days. The authors did not prove that the growth depressing effect of bentonite was due to vitamin A deficiency since they did not feed additional vitamin A to counteract it. In their studies with carotene in the diet (40,000 I.U. per kg.) the feeding of up to 3 percent of bentonite did not affect growth of rats (undisclosed age and weights) but did affect liver storage of vitamin A in 14 day experiments. It is possible that growth rates would have been reduced if the rats had been kept on experiment for a longer period.

Bentonite caused increased liver size in animals fed diets containing carotene but, for some unknown reason, an opposite effect was produced in rats fed diets containing vitamin A and bentonite.

show that the deficiency may be overcome by larger amounts of vitamin A and that the particle size of bentonite is involved in the production of deficiency signs. Laughland and Phillips made no mention of the particle size of the bentonite used in their studies.

Our data are not extensive enough to describe adequately the exact mechanism by which bentonite produces vitamin A deficiency. However, it is evident from the results of the vitamin A assays of the diet that at least part of the destruction of vitamin A by bentonite may occur directly in the diet even before it enters the digestive tract. It would appear that the presence of fat in the diet was in some manner associated with the destructive action of bentonite. This requires confirmation with a greater number of chicks.

The results of our studies demonstrate that caution is necessary in the use of bentonite in experimental synthetic diets with regard to the stability of vitamin A. It should be stressed that no evidence was found that the level of bentonite used in commercial feeds (1 to 2.5 percent) was in any way deleterious.[2] However, since a higher level of bentonite (20 percent) resulted in growth depression when added to a practical diet and because of the known adsorptive properties of bentonite for vitamin A, it is important that special attention be given to the level or source of vitamin A in poultry feeds containing bentonite of small particle size.

SUMMARY

Bentonite, a naturally occurring clay with adsorptive properties, caused a deficiency of vitamin A in chicks when fed at a level of 2 to 3 percent, or higher, in a synthetic diet otherwise containing ample amounts of vitamin A in unstabilized form. Both sodium and calcium bentonites were active in this regard. All signs of deficiency were prevented by the use of stabilized vitamin A, by higher levels of vitamin A or carotene, or by injected vitamin A.

It was found that the particle size of the bentonite was a factor in the development of vitamin A deficiency signs. When the bentonite was 90 mesh or coarser, no untoward symptoms were noted unless very high levels were fed (20 percent). The presence of fat in the synthetic diet may also have been a factor in the destructive action of bentonite.

Chemical analysis of the synthetic diet indicated that there was appreciable disappearance of vitamin A in the presence of bentonite.

No evidence was found that bentonite was not acceptable for use in practical rations at the levels commonly used in pelleted feeds (2.5 percent or less). At a much higher level (20 percent) growth and feed efficiency decreased.

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THE INFLUENCE OF ABSORBENTS (BOLUS ALBA) ON GROWING RATS WITH CHRONIC VITAMIN D_3 POISONING:

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The Behavior of Bones and Endosecretory Glands, Especially of the Parathyroid Gland, Examined by Chemical-Analytical, Histological and Roentgenological Methods.

Introduction and Posing of the Problem

Bolus alba is able in vitro to adsorb different substances which are pysiologically important nutrients. This "in vitro" adsorption is transferred to "in vivo" processes as a gauge for medications. Naturally, in oral administration, the adsorption process is subject to changes due to the different conditions in different areas of the digestive tract, so that the adsorption force measured for different substances in vitro is no more physiologically valid.[3]

Generally, bolus alba has no influence on the normal resorption of nutritive and active substances in rats, as determined in metabolism and growth tests with balanced diets.[4] Quite different is the action of "active" adsorbants with intramicellar structure, like activated charcoal which, among others, may influence the resorption of vitamin D_3 considerably.[4]

Earlier tests on adult rats established the possibility of inhibition of resorption of high doses of vitamin D_3 by oral administration of activated charcoal. This reduction of resorption could not be proven with bolus, an increase of resorption was even suspected. As is well known, vitamin D_3 poisoning in rats leads to growth inhibition partially caused by diminished absorption of nutrients. In adult rats, the result is radical weight loss under reduction of the fat deposits and finally cachectic appearances with lethal outcome. It is possible to postpone the lethal outcome of vitamin D_3 poisoning by intermittent high doses of vitamin D_3, and so to arrive at differentiated weight differences of the test animals corresponding to the vitamin dosis.

The absence of major fat deposits in growing rats and the higher metaboli

activity during growth permits only doses up to a maximum of 10,000 IU vitamin D_3 pro day, adjusted to the type of food with higher vitamin D_3 doses in permanent tests and under avoidance of larger lethal losses during the first 10 weeks of life. Up to such threshold values for vitamin D_3, the growth development of the test animals occurs within the frame of the vitamin D_3 dosis.

The significance of such growth determinations is always the result of a summation. Only by following the individual fates of the test animals can we assure ourselves about the validity of the test results.

The question of the alimentary influence of vitamin D_3 poisoning by bolus alba therefore requires multiple test methods and confirmation by the organ test.

In a previous paper we had already reported about the development of bone changes in growing rats with chronic vitamin D_3 poisoning and had related these changes to determinations of mineral components, to the organic content, and to the X-ray picture. This report will deal primarily with a parallel test series which had be poisoned with different doses of vitamin D_3 with the admixture of bolus alba.

For the histological evaluation of the test material in view of an alimentary influence of bolus alba on the vitamin D_3 poisoning, bone tissue and endosecretory glands, especially the parathyroid gland (PT) were of special interest. We were immediately faced with the difficulty that it is impossible to judge quantitative growth changes objectively in the histological picture, because of the multiplicity of the tissue components, or that they can only be roughly estimated, and that the only objective clues for the histologist are qualitative morphological changes.

This experience was validated already in the first observations of histological preparations from bone tissue. Any objectivation of quantitative changes, either by measuring of osteoid seams or of growth zones of the metaphysis foundered because of the individuality of the material. We therefore had to limit ourselves to the qualitative-morphological evaluation of the

bone tissue and used the development of bone changes described in the earlier work as basis.

An evaluation of the PT, however, has more hope for success, both quantitatively and qualitatively, especially since a change of the structure can be expected as a consequence of hypervitaminosis D_3.

A direct effect of vitamin D_3 on the PT is not likely; the research of Demole and Tietze and Sander had shown that vitamin D influences the calcium content of the blood, and vitamins D_2 and D_3 that of the bones, even in the absence of the PT, therefore work independently from the glands. But an indirect influence with corresponding histological changes of these endosecretory glands is likely, considering the close relations to the Ca and P metabolism and a massive disturbance as that certainly caused by the experimental vitamin D_3 hypervitaminosis. Observations in human mineral metabolism disturbances like rickets and corresponding animal experiments had shown that the PT reacts to such stimuli with a measurable enlargement which is also expressed in the cell structure. These growth changes could already be unambiguously determined and their result functionally evaluated by our earlier methods.

But there is another method for the functional evaluation of the PT. Engfeld shows in his very careful research that the size of the nuclei of the PT permits conclusions on the state of functioning of this organ. This work is based on research by Jacobi and others who deal extensively with the question of nuclear size and cell function. Recently, Hanssler also used this method to examine the influence of vitamin D on the PT.

These methods show a way to approach the special problems of this research also with hisologically quantitative methods. The histological observation of other endosecretory glands and the organs was to supplement the research and to add to the observations of the total changes a few specifics on the

mechanism of hypervitaminosis D_3.

Test Methods

The animal material used in this research includes a total of 180 albino rats of our own inbreeding over several years at the age of 30 days. The body weight of the rats at the beginning of the test lay between 52 and 56.5 g. 6 test groups of 15 male and 15 female animals each were formed by equal distribution of the animal material by weight. In the DPT group, the parathyroid glands (PT) had been removed by the previously used method.

The rats received daily orally by means of a probe 0.1 ccm vitamin D_3 solution (ethanol), the controls the same dosis of equal concentration ethanol. At the end of the third test week, the vitamin D_3 application was interrupted on two days and all animals received 0.1 ccm olive oil with 100 IU vitamin A (Vogan) to lengthen the survival time of the poisoned rats. The oral administration of 0.8 g bolus alba DAB 6 in the B-groups was achieved by withholding feed from all rats from 6 P.M. and giving to the animals in groups 5 B and 10 B in the morning after administration of the vitamins a mixture of 2 g feed and 0.8 g bolus alba which was consumed within a short time.

The diet, consisting of 10 teasp. oat meal, 1.5 teasp. German fish meal, 1 teasp. soybean meal, and 0.5 teasp. feed yeast which was available to the rats during the day ad libitum contained 0.882% Ca, 0.176% Mg, and 0.949% P in the dry solids and was, considering the phytate content, still adequate in respect to Ca and P. The yeast addition helped assure the supply of B-vitamins. Drinking water could be taken ad libitum during the feeding periods. During the last 3 days of each test week, the rats were weighed before eating, and corresponding to the average determined weights the two lightest and the two heaviest animals of each test series and sex killed and the necessary organs removed. Femur and tibia were cleanly prepared and subjected to the previously reported methods of analytical, histological and roentgenological determinations.

Test Results and Discussion

Weight development of the rats

The development of body weight in the test series is graphically represented for the single test series in figure 1.

In regard to our topic, no major difference is indicated in figure 1 between the weight development of the vitamin D_3 poisoned test series fed with or without bolus alba. The weight differences between series 5B and 5N and between 10B and 10N are not signficiant. No significant differences between the sexes can be found in the comparable test series either, but only in the control group with vitamin D_3 supplement, which are here without significance. The initial weight loss in the DPT series as compared with the equally dosed test series is likely to be a consequence of the operation of these animals. Significant is only the good recovery of these rats from the third test week on.

Beginning with the fourth test week, there is a pronounced weight increase in all vitamin D_3 poisoned animals. Here, the weight increase in the fourth week is stronger in the low dose series (5N, 5B, DPT) in spite of unchanged vitamin D_{3doses} (7500 IU) than in the initially higher dosed series 10B and 10N. This must be taken as a consequence of the two day interruption of the vitamin D_3 administration and the vitamin A administration on these days, but might also coincide with a natural growth increment natural in a growing animal. The latter, however, is highly unlikely. In the fifth test week, the vitamin D_3 poisoned animals suffer again a weight loss which is more obvious in the low-dosed animals.

The development of body weight leads to the initial suspicion that, in all grades of vitamin D_3 poisoning, no essential difference exists between the test series with or without bolus feeding, as found in the adult rats. The slightly different weight development of these groups in the critical third test week is definitely a consequence of the differential feed consump-

tion. This was steadily controlled during the course of the test. During the third test week, e.g., the average feed consumption of the 5N group was 5.55 g compared to 6.2 g in the 5B series; in the 10N group, 6.1 g, in the 10B group, 5.5 g pro animal and day. Feed consumption and weight development, however, cannot always be correlated in this experiment, since in chronic vitamin D_3 poisoning a disturbance of utilization may occur in the metabolism which might cause a certain compensation for the differential feed consumption without being determinable by weight changes alone in this experiment. It should not be forgotten that the average weight of the rats in the vitamin D_3 treated series moves, during the first 3 test weeks, only slightly from the initial weight at the beginning of the test,and that in the higher dosed groups the weight may even fall below the initial weight. The autopsy of these rats indicated that they had no fat deposits or only very minimal ones. Therefore, finer differences should not be read from the differences in the weight development of the different test series due to Alimentary influence of bolus alba on vitamin D_3 poisoning in growing rats.

The lowered activity, the increased appearance of external symptoms of vitamin D_3 hypervitaminosis on the eye of the bolus fed test groups lead to a suspicion of a difference from bolus-free rats, but this could not be proven.

Mortality

In spite of preventive measures to avoid lethal effects by sufficient vitamin B supply and transitory vitamin A doses, as well as by the adaptation of the vitamin D_3 dosage, there were single losses from the third test week on. Several rats could still be killed during the agony and included in the examination during the weekly removal. These animals are included in the following survey of the mortality in the single test series. We can ignore the losses in the DPT group as a consequence of the operation during the first test week.

In the test series fed bolus alba the number of lethal losses is highest as compared to the corresponding series of vitamin D_3 poisoned rats. This we take as an indication for the different behavior of the bolus alba fed animals versus all other comparable test animals. Even though the loss of these animals influences the calculation of the weight development only slightly and hardly disturbs the result of the total evaluation, the average weight of the 5B and 10B groups is slightly improved by the loss of 4 animals each in the last test week, so that an approach of the weights of the 5B and 5N groups should have been suspected at this time, had the animals survived.

Change on the bones

In the preceding paper, we had already described extensively the changes on the bones caused by vitamin D_3 hypervitaminosis. Here, we want only to point to the changes that are necessary to an understanding of the differences between the single test series and their evaluation.

The histologically and roentgenologically recognizable processes occur most clearly in the metaphysis of the long tubular bones and happen in certain phases within the test period. They start with an increasing calcium deposit into the original spongiosa with simultaneous osteoid formation around the little bone joints of the metaphysis. At the same time, a growth inhibition in the epiphysis line is recognizable, which leads to growth stoppage. The calcium deposit increases simultaneously and reaches also the osteoid, with the result of osteosclerosis. In the further course with unchanged poisoning new growth slowly starts and the osteosclerotic process comes to a halt. The bone masses formed up to now are decomposed by resorptive processes, the dense spongy bone is loosened by fibrous marrow with numerous osteoclasts. This picture, which resembles a localized osteodystrophia fibrosa, we consider to be a state of compensation, since now the organism seems to be able to paralyze the vitamin D_3 effect and at the same time starts to disassemble the osteosclerotic, excessively formed bone by resorption, to grow

again, and thereby to normalize the bone structure.

As we initially mentioned, it is impossible to differentiate these changes in their course in each phase quantitatively by histological methods. But we are able to point out in the last phase (5th week) the differences in the formation of the state of compensation which we studied especially in the series with low vitamin D_3 dosage.

We counted the frequency of this histological characteristic of compensation in the 5th test week,and came to the following result:

In examining the above compilation, we notice that - with the exception of the parathyreoprived animals (DPT) - the 5N group shows this characteristic more frequently and much more distinctly. In a comparison of the 5N group with the 5B group, the fully expressed state of compensation is most frequently represented in group 5N based percentually on the number of surviving rats. The difference between rats feds with and without bolus alba becomes still clearer in a comparison of the 10N series with the 10B series. The lethal losses are here only partially included. In comparing here already the result of this count with the above mentioned mortality in the single test series, a conformity between the results is found at least in as far as there are differences between the bolus fed rats and rats with equally high vitamin D_3dosis which indicate more severe damage to the bolus fed animals.

In the final phase of the test, the histological picture is, beyond that, largely dominated by a distinct osteosclerosis of the metaphysis, mainly in the 10B rats. The bone parts appear here especially high in calcium and dense. But a certain loosening of the spongiosa can be recognized on the average in the 10N series. The calcium deposit does no more appear to be so intense. Obviously and definitively compared to these two groups, the bone of the 5N and 5B series is loosened and its calcium density diminished.

This corresponds to the increased appearance of the resorptive process in these groups.

In picture 2, we have juxtaposed the X-ray pictures of the distal femur head and the proximal tibia end of 5 each of approx. equal weight male rats of test series 10N and 10B. The roentgen positive is enlarged approx. 3X linear.

Since these bones were photographed in a single exposure, we are entitled to draw conclusions on the degree of calcification of the bones from the density of the shadow. Only in the first bone in the 10B series can a slight lightening be noticed, corresponding to the above described loosening of the spongiosa of the metaphysis, while in the bones of the 10N series (from left 6-10) this lightening of the metaphysis is more or less well recognizable in all animals. Also, the zone of primary calcification (directional joistlets) in the tibia of the 10N series is clearly broadened, while in the tibia of the 10B series (from left 1-5) it is relatively small and distally set off against the lighter spongiosa. Conditions for the female rats of these series are similar. It would go too far to enumerate here the roentgenological differences between the test series with lower vitamin D_3 dosage which logically confirm the above findings on the difference between the two discussed test series.

Since the compensation state indicates the equalization and acclimatization of the organism to vitamin D_3 poisoning, or permits the conclusion about its intensity, it can be concluded that the rats under 5N conditions can overcome their hypervitaminosis the easiest and can slowly compensate for it, while this equalization in the 5B rats is present but not so effective. Under the conditions of the 5B group, the vitamin D_3 effect must therefore be stronger and longer lasting. Corresponding to this evaluation, in initially twice as intensive poisoning (10N and 10B groups) histological changes

in the sense of a compensation are only hinted towards the end of the test, with the 10B animals marked by a stronger stress. Therefore, the total picture shows a step-like effect which begins with the 5N series and ends with the 10B series.

Corresponding results are found in the chemical analysis of the long bones. We determined fat free dry solids, ash, calcium, and phosphorus in tibia and femur. Here, we limit ourselves to the ash content of these bones. We were unable to derive any constructive relations from our Ca and P determinations, the Ca:P ratio of the bones indicated no differciable differences which might have made a contribution towards the clarification of the problem.

Since D_3 hypervitaminosis leads to growth inhibition, we tried wherever possible to consider this in the observation of the mineral content. The development of the body weight of the rats did not appear to be the right standard since the animals partially fell below their initial weight in the test series, which is an expression of absorption of substance and not stoppage of growth. We chose therefore as a standard of comparison for growth the length of the tibia, since it cannot be assumed that the bone would be able to participate in changes of substance at the same rate as the variations in body weight.

The bone is able to adjust its volume with larger or smaller amounts of substance by changes in its structure. With equal amounts of substance, organic or inorganic, different amounts of water may be used and thereby different growth in length achieved. A comparison of absolute values for bone substance therefore does not lead to satisfactory results for the degree of structural quality at a given stage of development and for the growth of developing bones. The ratio of inorganic material to the fat free total solids of the bone leads to better understanding of its structural change without sufficiently considering the factor of growth.

The ash content of the normal bone increases corresponding to its growth in length. In our young bones with the capacity to grow, the development of the ash content follows, true to tendency, largely the development of the body weight of the rats. When, according to our earlier described theory, vitamin D_3 hypervitaminosis is characterized by alternating, histologically observable structural changes of the bones, and at the same time more or less severe growth inhibitions take place, as compared to the controls, this growth has to be represented by a special factor in the comparison of bone components determined by analytical chemistry.

It seemed interesting to relate the length of the tibia of the individual to its ash content. The length of the tibia was measured in 1/10 mm from identical points of the condylus fibularis to the malleolus tibiae of the single bones and this value used, expressed as cm, in the calculation of the ratio: "% ash in the original material to length of the tibia". Figure 3 shows the median values of this ratio graphically represented for the test sequence and the single test series.

The chemical-analytical values for the tibia correspond to those for the femur. There is no difference in tendency between these two bones as to changes due to vitamin D_3 hypervitaminosis. We only used the percent ash in the dry solids of the femur to show the correlation of analytical values with the histological picture for the explanation of differences between the test series. We used the tibia for the measurement of length since it is more suitable than the femur. Figure 4 shows graphically the median values of this ratio for the test sequence and the single test series.

Let us start our discussion of the different behavior of the test series as to bone development with the second week of the test. According to the initially discussed phase progress of the bone changes during vitamin D_3 hypervitaminosis, the rat series 5B and 10B, fed with bolus alba, show a

definite increase of ash; tibia length value compared to groups 5N and 10N, caused in part by increased inhibition of the growth of the bone (Fig. 3) in part by increased mineral deposits. The latter is clear from figure 4 where we show the ash content of the fat-free dry femur solids. We want to indicate here that histologically the picture of osteosclerosis exists at this stage in all comparable test groups. According to this, growth inhibition as well as degree of calcification are more pronounced at this stage in the B-groups than in the comparable N-groups. These previously discussed histological differences between the test series find here their quantitative confirmation.

When looking at conditions in the fifth test week, at the end of the test, we find the histological results, here too, quantitatively confirmed. While, according to figure 4, hardly any differences exist between the ash content of the fat-free dry solids of the single series, we find these distinctly when looking at the ash:tibia length ratio (fig. 3). Especially the 10B group shows severe growth inhibition compared to all other groups. A differenciation of the low dosis series is also possible in this phase when considering that the vitamin A administration in the third test week could influence the tibia length. We will discuss this possibility later.

It should be mentioned that a comparison with the normal animals is out of place here, even though the B series, especially 10B, showed a similar tendency of the mineralization process (Figs. 3 and 4) as the normal group in the fifth and in the first two test weeks. These groups cannot be compared since the process of ossification is histologically fundamentally different.

The discussed bone changes indicate unambiguously that the effects of vitamin D_3 hypervitaminosis is alimentatively influencable by feeding with bolus alba.

Changes of the Parathyroid Gland (PT)

The evaluation of the PT was performed by two methods. The first method proceeded from our own earlier observations that parathyroid glands increase with continuous functional stress and the increase of their volume runs parallel to the hyperfunction and its effects. It is therefore possible to draw conclusions from this change of size to the functioning of the glands.

For this type of evaluation, the PT and/or the neck organs of the test animals after embedding were in the usual way sliced into 5 μ series and each 5th section mounted, dyed, enlarged 100X and the circumference of the PT projected by means of a drawing apparatus on cardboard of equal thicness, outlined,and cut out. The cardboard leaflets obtained from a single series of PT were weighed together. We did not obtain absolute values, but "relative" ones which we could compare with those of the normal animals. The results are shown in the following table and in figure 5.

Fig. 5 indicates that the weight of the PT of the control animals increases initially at an even rate. After the 4th week, when the normal animals have already reached a weight of 150 g the weight of the PT increases only little. The female animals show a lower PT weight in the fifth week. In the test groups poisoned with vitamin D_3 the weight curves run rather evenly parallel to the abscissa in the total picture, with the exception of some minor variations, compared to the shape of the curves from the normal animals. Clear differences are, however, visible already in the second week, in that the more severely poisoned rats show a higher reduction of their PT volume. In the third week, the PT weight of the 10B rats arrives at a minimum which lies below that of all other groups and which indicates that these animals, in agreement with our bone results, have been most severely damaged. In the fourth test week, there is a slight increase of weight in all series, especially distinct in the 10B rats. At this time, the animals received for

2 days vitamin A as an antidote to vitamin D_3 as their weight loss became too large and we feared premature losses, and this lead to temporary improvement. The remission is expressed in the increased volume of the PT.

At the end of the test the same gradation appears as observed on the bone. The 5N rats have the relatively highest PT weight. With steadily lower PT values follow the 5B, the 10N, and then the 10B animals. The intensity of the D_3 poisoning therefore increases in this sequence and is expressed in the reduction of the PT.

The second method for the valuation of the PT is based on the recognition that the nuclear size of a parenchymatous organ indicates something about the state of functioning of the cell, where increase of the nucleus volume indicates increased activity and vice versa.

This method has recently been frequently used for the evaluation of endosecretory glands. The method was tested on PT by Engfeld and von Hanssler. Engfeld studied mainly the relations of the parathyroid glands to the pituitary gland, and Hanssler to vitamins D_2 and D_3. The latter results were only published recently, after our experiments had been concluded and evaluated. He finds during daily administration of approximately 30 γ vitamin D_2 or D_3 to rats weighing 50 to 70 g after 6 to 13 days stoppage of PT activity, expressed in reduction of the nuclei. It seems remarkable to us that the control animals as well as the test animals of this author do not gain weight during this time, according to the weight tables. Engfeld too was able in his thorough research to inhibit or increase the PT function by suitable conditions and to see the corresponding increase or reduction of the nuclei.

The size of the nuclei may be determined by two methods. Either the diameter is measured and the volume calculated from it, or the optically largest nuclear surfaces showing up in the section are determined by planimetration.

The first method is only applicable to uniformly round nuclei. When they assume ova shape, two diameters have to be determined and the volume calculated from them. This would be true for the PT of the rats, where the nuclei are predominantly oblong-oval. But exact examination of nuclei which appear, at first sight, to be round, indicates that they are by no means uniformly round. In practically every measurement of nuclei the problem is which diameter to use. The possibility of error is considerable and is further increased by the subjective judgment of the observer, and this error is raised to a higher power by the calculation of the volume. But this method is still frequently used in the evaluation of the adrenal glands under the wrong assumption than the nuclei of the rind are round, and is alsoused by Hanssler for the PT where he measures two diameters.

We decided in favor of planimetration after thorough examination of the methods, especially since the research by Engfeld had been made by this method and made a convincing impression upon us. This evaluation, however, is not free of errors either, especially as not the volume but only the area are measured and as the sectioning of the histological preparations does not always pick up the largest diameter of the nucleus. But these subjective and objective sources of error are reduced to a bearable minimum.

The single 5 μ thick PT sections are magnified 1300 times, their circumference drawn on thin cardboard and then measured with the planimeter. As the areas are small, we work with a short planimeter arm (40 mm) and thereby achieve larger deflections. The nucleus is traced three times, the total divided by three. The error of this method is 2%, therefore insignificant.

Two animals from each series, one each with the highest and the lowest body weight were evaluated and 200 nuclei per animal, a total of 12,000 measured. The numbers obtained represent not absolute, but relative directly read values. The 2 evaluated animals of one test week, 400 nuclei, are com-

bined and the frequency distribution of the classes of magnitude of the nuclei placed in a coordinate system.

We intentionally neglected to make a logarithmic or percentual compilation of the distribution curves, since by this method of representation nothing additional could be achieved scientifically and since many differences are not clearly expressed in our material.

Fig. 6 shows the nucleus curves of the normal animals. Here it is conspicuous that with increasing growth of the rats, the peaks of the curves are displaced towards the left, which means that the nuclei become smaller. In the fourth week, however, the curve returns once more above the initial value. These shifts are statistically significant. The decreasing nuclear size of the PT with increasing growthmay be due to the fact that the bone development slowly decreases in intensity with decreasing Ca turnover and therefore lowered demands on the PT. Engfeld could make similar observations on growing rats. Striking is the interruption of the decrease in size of the nuclei in the fourth test week, with a considerable enlargement taking place, so that the peak of the curve shifts by six units to the right. At this time, we have to explain this occurrence with the vitamin A administration, but we have to leave it undecided how to explain this effect in detail. From the result alone it could be concluded that the PT are induced to a considerable functional increase, that the Ca:P metabolism is influenced in a direction calling for increased parathormone production. We will return to this question later.

The reference to the fluctuation of the nuclear size during the growth phases seems especially important in order to express immediately which factors normally influence the size of the nuclei of rat PT, and that slight differences have to be judged with care even where the statistic calculation of the differences appears to be significant because of the large number of measurements.

In figs. 7a and be we have compiled the nuclear curves of test series 5N

and 5B, those series which initially received the low vitamin D_3 dosis. For comparison we added an average curve for the normal animals and omitted the female animals of the fifth week, to avoid unnecessary confusion and give a better survey. In comparison with the normal animals, the curve of the 5N series becomes increasingly steep beginning with the first week, with the peaks moving toward the left until this displacement of the curve becomes reversed in the third week. In the fourth test week, the curve moves one unit to the right, analogous to the normal animals, which occurs in all rats and was also indicated by the transitory weight gain of the PT.

The nuclei of the PT, according to the curve, therefore become smaller with increasing vitamin D_3 poisoning and their form more uniform, a fact that we will be able to demonstrate later clearly in the histological pictures. The peaks of the curves diminish from 14 units to 9 and 10 and increase in stages from the frequency value 40 to 75 up to the third week.

The nuclear curves of the 5B rats are similar. The difference to the 5D animals consists in the fact that the displacement to the left proceeds one unit further and reaches the value 8, and that the height of the curve exceeds the frequency value of 80. Therefore, the diminution of the nucleus and its uniformity are more strongly expressed than in test series 5N. Again, we see a confirmation of the increased toxicity of vitamin D_3 during bolus alba administration.

Fig. 8 which represents the behavior of the PT nuclei of test series 10N and 10B clearly shows the left-displacement and the increase in height of the curves. Compared to the preceding series, this did not occur from week to week but was completely expressed in a single leap from the first to the second week.

In detail, it appears significant that the nuclear curves of the 10N rats did not displace themselves as far to the left as those of the 5B animals.

Their peaks lie in units 9 and 10, they therefore resemble far more those of the 5N series, while otherwise the curves of the 10B series rather resemble those of the 5B animals as to left-displacement and height. This evaluation expresses less the difference in dosage than the influence of the bolus alba.

Therefore, during vitamin D_3 poisoning the nuclei of the PT are likely to decrease as an expression of inhibition of function, where the vitamin D_3 dosage within the given limits causes no clear difference of effect, but the addition of bolus alba to the food causes a significant difference in the sense of an increased hypervitaminosis.

We could have been satisfied with the evaluation of the PT, especially since it agreed very well with the findings on the bones, and yet we asked ourselves if the reduction of the total volume of the PT and its nuclei expressed a true functional inhibition or represented a reduction through general damage to the organism.

To follow this problem further, the nuclear size was put into relation with the total volume of the PT and thereby in a certain way a nucleus: plasma relation achieved. Only such an evaluation should approach an inclusive evaluation most closely, since the nuclei seem to participate in the cell function together with the cytoplasm. A resolution of the nucleus-plasma relation in this sense has frequently been attempted. It is a methodically difficult problem which has not been satisfactorily solved to this day. We do not want to go into details. The present type of relation is not irreproachable in its compilation. But it appears to be instructive and, combined with the histological picture, to touch the essentials of our problem.

The curve for this nucleus-plasma ratio is drawn as a curve in fig. 9. We want to limit ourselves in its discussion to the results on the normal animals and the 5B and 10B groups, in order to avoid unnecessary complications.

In the normal animals the nucleus-plasma ratio decreases quickly with increasing growth, to remain from the third week on on the same height with minor fluctuations. The 5B rats shows a continuous slight decrease of the ratio. In the 10B rats, the relation remains approximately on the same height, but shows a strongly changeable tendency which is reversely in remarkable agreement with the ash-tibia length ratio (fig. 3). This reversal may be due to the change of size of the nuclei or to variation of the protoplasm volume, just as the ash-tibia length ratio reflects the function of the mineralization and the growth of the bone. This comparison of absolute values indicates that both parts are involved in this fluctuation.

It is now interesting to pursue these fluctuations in the histological picture of the PT, which reflects the course of the curve (fig. 9) clearly. We chose the above mentioned three test series and in fig. 10, we juxtapose the histological picture of the PT in the first, third, fourth, and fifth week.

In the series of the normal animals (fig. 10) it is clearly shown that the nuclei are smaller in the third test week than in the first, then increase slightly in the fourth week, and become smaller again towards the end of the test. The course of the nucleus-plasma relation (fig. 9) appears therefore essentially caused by the behavior of the nuclei. In series 5B (fig. 10, center), the nuclei are, in comparison to those of the normal animals, of considerably reduced volume and instead of oval and oblong now mostly rounded, densely placed and characterized by a constant relation to the cytoplasm corresponding to the curve in fig. 9.

In the 10B series, (fig. 10,bottom), the change of the nucleus-plasma relation is impressively obvious. The first picture still corresponds to the overlying row of the 5B group. In the third week, the nuclei are rounded, densely placed and the cytoplasm has largely disappeared. The nucleus-

plasma relation (fig. 9) rises steeply corresponding to the curve in favor of the nuclei, though from an absolute point of view they lose considerably in magnitude. In the further course of the test, the cytoplasm as well as the nuclear volume of this series increases considerably in mass, to return in the fifth week to the same state as in the third. This alternating behavior, and/or the obvious functional recovery during the fourth test week ought to be due in the 10B series to the above mentioned administration of vitamin A and the relief due tothe skipping of the vitamin D_3 administration for two days.

It is obvious that only this type of observation permits a comprehensive insight into the histologically functional changes of the PT, that the curves by themselves may permit a rough survey but by themselves do not permit to recognize the true conditions. In reference to our present topic, this evaluation assures fully the result of our previous findings.

The Relation of the Changes of the Endosecretory Glands to the Vitamin A and D Doses

The symptoms of vitamin D hypervitaminosis are influenced and diminished by vitamin A, vitamin B complex, and partially by as yet unknown fatty additives. The experimental experiences are known facts, but is still largely unknown if these vitamin D antidotes in the hypervitaminosis under discussion are only of symptomatic nature as consequences of the vitamin D_3 poisoning or have a fundamental relation to the specific vitamin D_3 effect on endosecretory glands and especially their influence on the mineral metabolism. The answer to this question would help to identify the mechanism of the physiological action of bolus alba in its alimentary influence on vitamin D_3 hypervitaminosis. The above mentioned results show clearly that bolus alba has such an influence.

In earlier tests with normal feeding and vitamin dosage, the adsorptive

character of bolus alba in the intestine could not be proved. E.g. in the growth test with low doses of vitamin A, no alimentary influence of this extramicellar adsorbent could be observed, and tests with low vitamin D doses did not succeed either. Only under conditions of vitamin D_3 hypervitaminosis an alimentary influence of bolus alba on mature rats could be shown which, however, did not correspond to the adsorptive character of this compound in connection with the composition of the diet. Since these tests had the same result with or without vitamin A admixture to the feed, the administered doses of vitamin A were in addition removed from the attack of the adsorbent, it must be assumed that the adsorption of vitamin D_3 is furthered by bolus.

An alternating effect between the vitamins can be excluded for the normal metabolism according to the research by Scheunert. In the toxic damage of this experiment we have to examine our results to find which changes of the organism can be considered direct consequences of the vitamin D_3 administrations and which have to be considered secondary effects. Without being able at this point to give a final survey about the subject, we have attempted to differenciate in this context at least between the effects of vitamin A and D, in order to further secure or organ tests.

Further on, the reported bone changes are certainly also connected to the function of the endosecretory glands via the Ca and P metabolism. Endocrinological research concerns itself mainly with the study of deficiency phenomena, after endocrine gland ectomy. We therefore included in our tests also a parathyreoprive test series, in order to observe the influence of the PT on the hypervitaminosis of the vitamin D_3.

The extirpation of the PT was performed by the usual, formerly frequently used method. The technique is described in these articles. Since in Vigantol poisoning no tetany can be expected as sign of the successful operation, the neck organs of the animals must be cut open in series and the success of the

operation controlled by microscopic examination of the series.

The histological bone changes show, as indicated in our earlier work, no fundamental differences to tther test groups. Rather, they are identical in the phaselike course of the bone processes and as to the frequency of the compensation process they correspond to the 5N group. Apparently, the missing of the PT hormone and its effect are largely obscured by the vitamin D_3 poisoning. However, in comparing the frequency of the compensation state in the PT group with the other test groups, it is obvious that all but one of the parathyreoprived animals develop resorptive degradation processes on the bone and more than half of them show the distinct state. In considering in addition the total histological picture, it must be stressed that the picture of osteodystrophia fibrosa is especially pronounced in this group of animals, that their sclerosed bone is most severely disintegrated and its calcium content reduced. The ratio of tibia length to ash content confirms quantitatively the histological results in a comparison between test series 5N and DPT. As a consequence of the operation, the development of body weight of the parathyoprived rats, and therefore to a certain degree the length growth of the tibia, is inhibited. Independent of this, the bone of the DPT group passes through the previously described phases of the bone changes. Compared to the 5N series, the percent ash content in the dry solids of the tibia, which we described in the previous paper, is slightly higher at the beginning of the test in parathyreoprived rats. This is also expressed (fig. 11) in the ratio ash-tibia length, where the value of this ratio initially exceeds that of the 5N series slightly.

As clearly indicated in fig. 11, however, the ash-tibia length value decreases severely from the fourth to the fifth test week, the time of the compensation stage, with now starting increased growth of the bone, as indicated by the absolute values for tibia length.

It is remarkable that these rats arrive at a compensation without the help of the PT. The results on the bone which characterize the differential behavior of rats with and without bolus feeding we have therefore to accept as mainly independent from PT.

Now, how do the other endosecretory glands behave? We parsued this question to get closer to the point of attack of vitamin D_3. In dissecting the animals we had observed a reduction in size of the testicles, a high grade atrophy of the thymus gland, and an enlargement of the adrenal glands. We also considered a disturbance of the pituitary gland in connection with the growth stoppage. A histological examination of the pituitary gland did not confirm this assumption. Histological observation of the testicles showed inhibition of the maturation but the formation of germ cells was retained and vital up to the formation of spermatocytes.

In contrast with this, the relation of vitamin D_3 hypervitaminosis to the adrenal glands is interesting since our test throws a certain light on the relation of these glands to the PT. It is known from the literature that adrenalectomy causes increased action of the PT hormone and elevation of the blood Ca. Chronic adrenal insufficiency may cause enlargement of the PT. PT extirpated rats show increased lipoid storage in the adrenal rind which indicates increased hormone discharge by the adrenal gland. In research on the behavior of kidney phosphatase in parathyreoprived and adrenalectomized rats we were able to point to a similar disappearance of the enzyme in this organ and interpreted the finding as further proof for similar, possibly supplementary, action of the two glands.

In the present test we have weighed the adrenal glands of all rats and have graphically represented the obtained values as percentage of body weight in fig. 12.

In comparison with the normal animals,the weight of the adrenal glands of

the vitamin D_3 poisoned rats increased clearly in the test series, with more highly poisoned animals showing this increase more definitely than those with lower dosages. This increase, according to present theory (Selye) should be considered a consequence of a chronic stress to which the adrenal gland adjusts. Formerly, we termed this behavior in connection with chronic cooling as "compensation stage". Histologically, it is combined with increased lipoid deposition in all layers of the rind. It is important to state that the adrenal gland of the parathyreoprived animals increases most in weight. Could it be that the adrenals step in for the hormone loss of the PT at least vicariously and thereby bring about the balance (compensation) more quickly than in the not parathyreoprived rats? This question has not yet been unequivocally answered in this result but could be most easily explained in this sense.

The thymus shows histologically complete emptying of lymphocytes in the rind, so that only the reticulum remains in the extreme case. It cannot be immediately said if this result conceals more than the consequence of general physical strain and poisoning. It might remind one of Nitschke's theory who suspects the presence of a calcinose factor in the lymphatic tissue. But we do not want to go into the details of this relation. There remain the findings on the other organs. Only the changes in the kidneys appear to be worth mentioning. Already macroscopically, they are conspicuous by their enlargement and pale yellow color which gives them the appearance of nephrosis. Microscopically, the lime deposition known from the literature in cases of vitamin D_3 poisoning is found. The lime deposits underneath the epithelium of the winding renal tubules in the form of layered balls which enlarge, compress and deflect the epithelium so that it mortifies and the lime particles enter the lumen of the tubules. They partially get stuck in the descending part and close the lumen so that the tubules above become considerably enlarged and partially decay under formation of small scars. The

changes resemble those from chronix lead poisoning that we described earlier. We did not think these findings suited for quantitative evaluation.

We did not obtain any special histological results on the thyroid glands. The influence of vitamin D on the thyroid function is known, and hypofunction of the thyroid can be offset by vitamin D administration. Participation of the thyroid in the vitamin D hypervitaminosis should not be theoretically excluded. The loss of body weight after high doses of vitamin D could possibly be imagned as being partially due to this gland, especially since we could not attribute the differential weight loss of the single test series alone to the differential feed consumption, and since earlier tests had not shown any influence of bolus alba on the digestability of the nutrients in the diet.

According to this we can therefore assume that the bone changes as well as the changes of PT and adrenal glands are an immediate consequence of the vitamin D_3. Therefore the differences between the different test series rest upon a direct promotion of resorption of vitamin D_3 by bolus alba. This promotion of resorption can be imagined purely mechanically as a consequence of improved surface distribution of the vitamin D_3 by the extramicellary adsorbent. The process of resorption is therefore preceded by an adsorption on easily accessible surfaces, while in intramicellary adsorbents inner surface adsorption inhibits the resorption by local inaccessability. Influence on vitamin D_3 hypervitaminosis by adsorption and lessening of resorption of vitamin A by bolus alba appears impossible as indicated by earlier tests with vitamin A and bolus. The vitamin A activity of the diet was too low to be able to influence the vitamin D_3 hypervitaminosis in any degree. The interim administration of vitamin A was removed from the influence of the adsorbent. Though it was sufficient, as we suppose,and as far as it is not also due to the two day interruption of the vitamin D_3 administration, to cause recovery

sumptoms in the PT and adrenals, but it was not sufficient to cover up the existing differences between the rats fed with and without the addition of bolus.

Other alimentary influences on the vitamin D_3 hypervitaminosis caused by bolus alba are theoretically possible but cannot be discussed in the frame of our experiment.

Summary

Growing rats were used to examine the alimentary influence of bolus alba on the picture of vitamin D_3 hypervitaminosis. Test objects were femur, tibia, parathyroid, thyroid, and pituitary glands, testicles, thymus, and kidneys. The tests were made by chemical-analytical, histological and roentgenological methods. The following results were obtained:

1. Over a test duration of 5 weeks, the bones showed varying changes which lead via the state of osteosclerosis with increasing calcification to a resorptive decomposition of the excessively formed bone. The latter changes are considered to be the expression of a compensatory process by the organism against the vitamin D_3 poisoning. Measured by the degree of calcification, the ratio % ash to tibia length, and the % ash content of the fat free dry solids of the bone, as well as the degree of osteosclerosis and the compensatory processes, an alimentary influence of bolus alba on the vitamin D_3 hypervitaminosis if found which can be considered to be due to improvement of resorption of vitamin D_3.
2. A functionally statistical evaluation of the total volume, the nucleus size, and the nucleus-plasma ratio of the parathyroid gland verifies this result. In detail, the state of functioning of the parathyroid glands of normal rats decreases with increasing growth. This decrease of function follows in vitamin D_3 poisoning the intensity of the poisoning. The func-

tional inhibition of the parathyroid glands can be canceled by administration of vitamin A.

3. Examination of the pituitary and thyroid glands and testicles in vitamin D_3 poisoning does not give any essential results. Among the other organs, only the kidneys are considerably changed by lime depositions and retention processes. Increasing poisoning leads to increasing atrophy of the thymus. The adrenal glands increase in size corresponding to body weight and degree of poisoning. Parathyreoprived rats are distinctive by special weight gain of the adrenals. Among these animals, the compensation on the bone takes place with special intensity without the help of the PT, as indicated by the histological picture and the ratio of % ash to tibia length.

So, there is an alimentary conditioned difference between the test series fed with and without bolus alba, which can be explained with the improved vitamin D_3 resorption after oral bolus administration, as clearly indicated by the behavior of the bone and the endosecretory glands.

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H. BRUNE und W. EGER

Der Einfluß von Adsorbentien (Bolus alba) auf wachsende Ratten bei der chronischen Vitamin D_3-Vergiftung:

Das Verhalten des Knochens und innersekretorischer Drüsen, insbesondere der Epithelkörperchen, untersucht mittels chemisch-analytischer, histologischer und röntgenologischer Methoden

Einleitung und Fragestellung

Bolus alba vermag in vitro verschiedene Stoffe, die ernährungsphysiologische Bedeutung haben, adsorptiv zu binden. Diese „in vitro"-Adsorption wird als Maßstab für Medikationen auf „in vivo"-Vorgänge übertragen. Bei oraler Zufuhr ist naturgemäß der Adsorptionsvorgang Wandlungen durch die verschiedenen Milieuverhältnisse des Verdauungstraktes unterworfen, so daß die in vitro gemessene Adsorptionskraft für bestimmte Stoffe physiologisch nicht mehr in Erscheinung tritt[3].

Im allgemeinen übt Bolus alba kaum einen Einfluß auf die normale Resorption von Nähr- und Wirkstoffen bei Ratten aus, wie sich im Rahmen von Stoffwechselversuchen oder von Wachstumsversuchen bei ausgeglichener Diät feststellen läßt[4]. Ganz anders verhalten sich stark „aktive" Adsorbentien mit Intramizellarstruktur, wie Aktivkohle, die u. a. die Resorption von Vitamin D_3 wesentlich beeinflussen können[4].

Bei erwachsenen Ratten wurde in früheren Versuchen[4] festgestellt, daß durch Aktivkohle eine Resorptionshemmung für hohe Dosen Vitamin D_3 bei oraler Applikation möglich ist. Diese Resorptionsminderung war bei Bolus-Fütterung nicht nachzuweisen, vielmehr wurde eine Resorptionsförderung für Vitamin D_3 durch Bolus alba vermutet. Bekanntlich führt die Vitamin D_3-Vergiftung bei Ratten zu einer Wachstumshemmung, die teilbedingt durch verminderte Nahrungsaufnahme hervorgerufen wird. Bei erwachsenen Ratten kommt es zu einem Gewichtssturz unter Abbau der Fettdepots und schließlich zu kachektischen Erscheinungen mit letalem Ausgang. Durch intermittierende hohe Vitamin D_3-Applikationen ist es möglich, den letalen Ausgang der Vitamin D_3-Vergiftung zeitlich hinauszuschieben und so entsprechend der Höhe der Vitamin D_3-Dosis zu differenzierten Gewichtsunterschieden bei den Versuchstieren zu kommen.

Das Fehlen größerer Fettdepots bei wachsenden Ratten und die höhere Aktivität des Stoffumsatzes beim Wachstum läßt der Kostform angepaßt mit höheren Vitamin D_3

Dosen im Dauerversuch unter Vermeidung größerer letaler Ausfälle während der ersten 10 Lebenswochen nur in Dosen bis maximal 10000 IE Vitamin D_3 pro Tag zu. Bis zur Höhe solcher Grenzdosen des Vitamin D_3 folgt die Gewichtsentwicklung der Versuchstiere im gewissen Rahmen der Vitamin D_3-Dosis[8].

Die Signifikanz solcher Gewichtsermittlungen ist stets das Ergebnis einer Summierung. Erst die Verfolgung des Einzelschicksals der Versuchstiere mit Hilfe mehrerer Untersuchungsmethoden kann uns Gewißheit über die Richtigkeit der Ergebnisse des Versuches geben.

Die Frage einer *alimentären Beeinflussung der Vitamin D_3-Vergiftung durch Bolus alba* bedarf demnach vielfältiger Untersuchungsmethoden und der Bestätigung durch den Organtest.

In einer vorausgehenden Arbeit[5] hatten wir bereits über *die Entwicklung der Knochenveränderungen* an wachsenden Ratten bei chronischer Vitamin D_3-Vergiftung berichtet und die Veränderungen *in Beziehung zu Mineralstoffbestimmungen*, zum Gehalt an organischer Substanz und zum Röntgenbild gebracht. In dieser Arbeit werden vornehmlich einige parallel geführte Versuchsgruppen besprochen, die unter *Zufütterung von Bolus alba mit verschieden hohen Dosen Vitamin D_3* vergiftet worden waren.

Für die *histologische Bearbeitung* des Untersuchungsmaterials *unter dem Blickwinkel einer alimentären Einflußnahme des Bolus auf die Vitamin D_3-Vergiftung* mußte das Hauptaugenmerk auf das Knochengewebe und die innersekretorischen Drüsen, insbesondere die Epithelkörperchen (EK) gelegt werden. Von vornherein war bei diesem Vorgehen die Schwierigkeit zu berücksichtigen, daß quantitative Größenveränderungen im histologischen Bild bei der Vielfalt der zu beurteilenden Gewebsanteile objektiv meist nicht oder nur in groben Schätzungen zu erfassen sind und sich nur die qualitativ morphologischen Veränderungen dem Histologen als objektive Anhaltspunkte zur Beurteilung des Geschehens darbieten.

Am *Knochengewebe* hatte sich diese Erfahrung schon in der ersten Musterung der histologischen Präparate bestätigt. Jede Objektivierung quantitativer Veränderungen, sei es durch Messung osteoider Säume oder von Wachstumszonen der Metaphyse scheiterten z. T. an der Eigenart des Materials. Wir mußten uns deshalb auf die qualitativ-morphologische Auswertung des Knochengewebes beschränken und konnten die in der vorangehenden Arbeit geschilderte Entwicklung der Knochenveränderungen als Grundlage nehmen.

Eine *Auswertung der EK* hat dagegen in quantitativer und qualitativer Hinsicht mehr Aussicht auf Erfolg, zumal eine Veränderung der Struktur als Folge der Hypervitaminose D_3 zu erwarten ist.

Eine direkte Wirkung des Vitamin D_3 auf die EK ist nicht anzunehmen; hatten doch die Untersuchungen von DEMOLE[8] und von TIETZE und SANDER[41] gezeigt, daß das Vitamin D den Calciumgehalt des Blutes und die Vitamine D_2 und D_3 den Knochen auch ohne Vorhandensein der EK beeinflussen, also unabhängig von den Drüsen arbeiten. Bei den engen Beziehungen zum Calcium- und Phosphorstoffwechsel und einer so massiven Störung, wie sie durch die experimentelle Vitamin D_3-Hypervitaminose sicherlich verursacht wird, ist aber ein indirekter Einfluß mit entsprechenden histologischen Veränderungen dieser innersekretorischen Drüsen wahrscheinlich. Beobachtungen bei menschlichen Mineral-

stoffwechselstörungen wie der Rachitis und entsprechende tierexperimentelle Untersuchungen hatten gezeigt, daß die EK auf solche Reize mit einer sicher zu erfassenden Vergrößerung reagieren, die auch im Zellbild zum Ausdruck kommt[9]. Diese Größenänderungen kann man nach der von uns schon früher geübten Methode eindeutig feststellen und den Ausfall funktionell auswerten.

Es gibt aber methodisch noch einen anderen Weg, um die EK funktionell zu beurteilen Engfeldt[14] zeigt in einer sehr sorgfältigen Untersuchung, daß die Kerngröße der EK Rückschlüsse auf den Funktionszustand des Organs zuläßt. Diese Arbeiten fußen auf Untersuchungen von Jakobi u. a.[23,21,2], die sich eingehend mit der Frage der Kerngröße und Zellfunktion befassen. Neuerdings bediente sich auch Hanssler[19] dieser Methode, um im besonderen den Einfluß des Vitamins D auf die EK zu untersuchen.

Mit diesen Methoden sind Wege gewiesen, um die besondere Fragestellung der vorliegenden Arbeit auch mit histologisch quantitativen Methoden anzugehen. Die histologische Betrachtung anderer innersekretorischer Drüsen und der Organe sollte die Untersuchung ergänzen und in der Gesamtbetrachtung der Veränderungen einen Beitrag für den Wirkungsmechanismus der Hypervitaminose D_3 im einzelnen geben.

Versuchsdurchführung

Das in der vorliegenden Arbeit verwendete Tiermaterial umfaßt insgesamt 180 Albinoratten eigener mehrjähriger Inzucht im Alter von 30 Tagen. Das Körpergewicht der Ratten lag zu Versuchsbeginn zwischen 52 und 56,5 g. Es wurden durch gleichmäßige Verteilung des Tiermaterials nach Gewicht 6 Versuchsgruppen zu je 15 ♂ und 15 ♀ Tieren gebildet. Bei der DEK-Gruppe waren die Nebenschilddrüsen (EK) nach der bereits öfters geübten Methode[9,10] entfernt worden.

Die Ratten erhielten täglich oral mit der Sonde 0,1 ccm Vitamin D_3-Lösung (Äthylalkohol), die Kontrollen dieselbe Gabe gleichprozentigen Äthylalkohol. Ende der dritten Versuchswoche wurde an 2 Tagen die Vitamin D_3-Applikation unterbrochen und an alle Tiere 0,1 ccm 01. oliv. mit 100 IE Vitamin A (Vogan) verabreicht, um die Überlebenszeit der mit Vitamin D_3 vergifteten Ratten hinauszuzögern. Die orale Zufuhr von 0,8 g Bolus alba DAB 6 in den B-Gruppen wurde dadurch erreicht, daß allen Ratten ab 18 Uhr abends das Futter entzogen und morgens nach der Applikation der Vitamine den Tieren der Versuchsgruppen 5 B und 10 B eine Mischung aus 2 g Futter und 0,8 g Bolus alba gereicht wurde, die in kurzer Zeit verzehrt wurde.

Die Diät aus 10 Tl. Haferflocken, 1,5 Tl. deutschem Fischmehl, 1 Tl. Sojamehl und 0,5 Tl. Futterhefe, die den Ratten tagsüber ad libitum zur Verfügung stand, war mit 0,882% Calcium, 0,176% Magnesium und 0,949% Phosphor in der Trockensubstanz hinsichtlich der Calcium- und Phosphorversorgung auch unter Berücksichtigung des Phytingehaltes noch adäquat[25] für Ca und P. Der Gehalt an Futterhefe half die Versorgung mit B-Vitaminen zu sichern. Tränkwasser konnte während der Futterzeiten ad libitum aufgenommen werden.

Über die *Einteilung der Versuchsgruppen* und den Dosierungsplan für Vitamin D_3 gibt folgende Übersicht Auskunft:

Versuchswoche	Versuchsgruppe					
	N Kontrolle	DEK parathyreopriv	5 N	10 N	5 B	10 B
					mit Bolus alba	
	Vitamin D_3 in 1000 IE					
1	—	5	5	10	5	10
2	—	7,5	7,5	15	7,5	15
3	—	7,5	7,5	15	7,5	15
4	—	7,5	7,5	7,5	7,5	7,5
5	—	7,5	7,5	7,5	7,5	7,5

In den letzten drei Tagen jeder Versuchswoche wurden die Ratten nüchtern gewogen und entsprechend den aus diesen Wägungen ermittelten Durchschnittsgewichten jeweils die beiden leichtesten und schwersten Tiere jeder Versuchsgruppe und Geschlechtes getötet und die zur Untersuchung erforderlichen Organe entnommen. Femur und Tibia wurden säuberlich präpariert und der analytischen, histologischen und röntgenologischen Untersuchung nach den bereits früher angegebenen Methoden[5] unterzogen.

Versuchsergebnisse mit Diskussion

Gewichtsentwicklung der Ratten

Die Entwicklung des Körpergewichts in der Versuchsfolge ist für die einzelnen Versuchsgruppen in Abb. 1 graphisch dargestellt.

Im Hinblick auf unser Thema ist aus Abb. 1 zunächst kein größerer Unterschied der Gewichtsentwicklung der mit und ohne Bolus alba gefütterten, mit Vitamin D_3 vergifteten Versuchsgruppen zu entnehmen. Die zwischen der 5 B- und 5 N- sowie zwischen der 10 B- und 10 N-Gruppe bestehenden Gewichtsunterschiede sind nicht signifikant. Ebensowenig lassen sich bei diesen vergleichbaren Versuchsgruppen signifikante Unterschiede zwischen den Geschlechtern ermitteln, die lediglich in der Kontrollgruppe ohne Vitamin D_3-Zufuhr bestehen und in diesem Zusammenhang nicht von Bedeutung sind. Die in der DEK-Gruppe im Vergleich mit den gleich hoch dosierten Versuchsgruppen anfänglich zu verzeichnende Gewichtsabnahme ist wohl als Folge der Operation dieser Tiere auszulegen. Auffällig ist lediglich die gute Erholung dieser Ratten ab der dritten Versuchswoche.

Mit Beginn der vierten Versuchswoche erfolgt bei allen mit Vitamin D_3 vergifteten Tieren eine verstärkte Gewichtszunahme. Dabei ist bei den niedrig dosierten Versuchsgruppen (5 N, 5 B, DEK) trotz unverändert bleibender Vitamin D_3-Gaben (7500 IE) der Gewichtsanstieg in der vierten Versuchswoche stärker als bei den anfänglich höher dosierten Versuchsgruppen 10 B und 10 N. Dieses Verhalten der Versuchsgruppen muß als eine Folge der zweitägigen Unterbrechung der Vitamin D_3-Zufuhr und der Vitamin A-Gabe an diesen Tagen angesehen werden, könnte jedoch auch mit einem bei wachsenden Tieren zwangsläufig erfolgenden Wachstums-

schub zusammenfallen. Letzteres ist jedoch äußerst unwahrscheinlich. In der fünften Versuchswoche kommt es bei den mit Vitamin D_3 vergifteten Versuchsgruppen wieder zum Gewichtsabfall, der bei den niedrig dosierten Tieren stärker in Erscheinung tritt.

Die Körpergewichtsentwicklung läßt zunächst vermuten, daß bei allen Graden der Vitamin D_3-Vergiftung kein wesentlicher Unterschied zwischen den Versuchsgruppen mit oder ohne Bolus-Fütterung besteht, wie dieser bei erwachsenen Ratten gefunden wurde[4]. Die leicht unterschiedliche Gewichtsentwicklung dieser Gruppen in der kritischen dritten Versuchswoche ist bedingt eine Folge des *unterschiedlichen Futterverzehrs*. Dieser wurde während der Versuchsfolge laufend kontrolliert. In der dritten Versuchswoche betrug beispielsweise der durchschnittliche Futterverzehr in der 5 N-Gruppe 5,55 g gegenüber 6,2 g bei der 5 B-Gruppe; bei der 10 N-Gruppe 6,1 g, bei der 10 B-Gruppe 5,5 g pro Tier und Tag. Futterverzehr und Gewichtsentwicklung können jedoch in diesem Experiment nicht immer in Beziehung gesetzt werden, da bei der chronischen Vitamin D_3-Vergiftung mit einer Verwertungsstörung im Stoffwechsel gerechnet werden muß, die hier einen gewissen Ausgleich für den unterschiedlichen Futterverzehr schaffen könnte, ohne in diesem Experiment durch Gewichtsänderungen allein erfaßbar zu sein. Es darf nicht übersehen werden, daß das Durchschnittsgewicht der Ratten in den mit Vitamin D_3 behandelten Versuchsgruppen sich in den ersten drei Versuchswochen nur wenig vom Ausgangsgewicht zu Versuchsbeginn entfernt, daß bei den höher dosierten Versuchsgruppen dieses Ausgangsgewicht sogar unterschritten wird. Die Autopsie dieser Ratten ergab, daß diese über kein oder nur ein sehr geringfügiges Fettdepot verfügten. Demnach dürften aus der Gewichtsentwicklung allein kaum feinere Unterschiede zwischen den Versuchsgruppen infolge alimentärer Beeinflussung der Vitamin D_3-Vergiftung durch Bolus alba bei wachsenden Ratten abgelesen werden können.

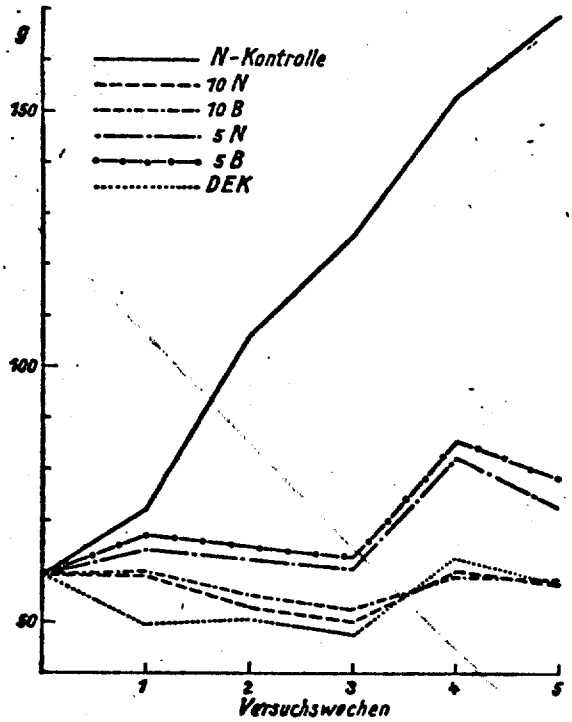

Abb. 1. Gewichtsentwicklung der Versuchsgruppen

Die verminderte Aktivität, das verstärkte Auftreten der äußeren Symptome der Vitamin D_3-Hypervitaminose am Auge bei den mit Bolus gefut-

terten Versuchsgruppen ließen einen Unterschied gegenüber den Bolus-frei ernährten Ratten vermuten, jedoch nicht beweisen.

Die Mortalität

Trotz vorbeugender Maßnahmen, durch ausreichende Vitamin B-Versorgung und vorübergehende Vitamin A-Gaben sowie durch Anpassung der Dosierung des Vitamin D_3 letale Abgänge zu vermeiden und die Versuchsdauer möglichst lange bei äußerst hoher Dosierung des Vitamin D_3 auszudehnen, kam es doch vereinzelt zu Ausfällen ab der dritten Versuchswoche. Einige Ratten konnten wir in Agonie noch töten und mit zur Untersuchung bei den wöchentlichen Entnahmen bringen. In der folgenden Übersicht über die Mortalität in den einzelnen Versuchsgruppen sind auch diese Tiere mit einbegriffen. Auf eine Wiedergabe der Verluste in der DEK-Gruppe als Folge der Operation in der ersten Versuchswoche können wir hier verzichten.

Todesfälle bis Versuchsende

	Versuchsgruppe					
	N	5 N	5 B	10 N	10 B	DEK
3. Woche	—	1	2	4	4	1
4. Woche	—	—	1	3	3	2
5. Woche	—	—	4	1	4	1
Insgesamt:	—	1	7	8	11	4

In den mit Bolus alba gefütterten Versuchsgruppen ist die Zahl der letalen Abgänge im Vergleich mit den korrespondierenden gleich hoch mit Vitamin D_3 vergifteten Ratten am höchsten. Wir sehen hierin einen Hinweis für das andersartige Verhalten der mit Bolus alba gefütterten Ratten gegenüber den übrigen vergleichbaren Versuchstieren. Wenn auch der Ausfall dieser Tiere die Berechnung der Gewichtsentwicklung nur geringfügig beeinflußt und das Ergebnis der Gesamtauswertung kaum stört, so wird doch durch den Ausfall von je 4 Tieren in der letzten Versuchswoche das Durchschnittsgewicht der 5 B- und 10 B-Gruppe um ein geringes verbessert, so daß eine Gewichtsannäherung der 5 B-Gruppe an die 5 N-Gruppe zu diesem Zeitpunkt bei Überleben dieser Tiere zu erwarten gewesen wäre.

Die Veränderungen am Knochen

Bereits in der vorangehenden Arbeit[5] hatten wir die Veränderungen am Knochen bei der Vitmin D_3-Hypervitaminose ausführlich beschrieben. Hier sollen nur die Veränderungen herausgestellt werden, die zum Verständnis der Unterschiede zwischen den einzelnen Versuchsgruppen und zu ihrer Beurteilung notwendig sind.

Die *histologisch und röntgenologisch erfaßbaren Prozesse* treten am deutlichsten in der Metaphyse der langen Röhrenknochen auf und laufen innerhalb der Versuchszeit in bestimmten Phasen ab. Sie beginnen mit einer zunehmenden Kalkeinlagerung in die ursprüngliche Spongiosa unter gleichzeitiger Osteoidbildung um die Knochenbälkchen der Metaphyse. Zu

gleicher Zeit macht sich eine Wachstumshemmung in der Epiphysenlinie bemerkbar, die bis zum Wachstumsstillstand geht. Währenddessen nimmt die Kalkeinlagerung zu und ergreift auch das Osteoid, so daß eine Osteosklerose resultiert. Im weiteren Verlauf kommt es bei gleichbleibender Vergiftung zu allmählich beginnendem erneutem Wachstum und zum Sistieren des osteosklerotischen Prozesses. Diese bisher gebildeten Knochenmassen werden nun durch resorptive Vorgänge abgebaut, der dichte spongiöse Knochen lockert sich durch fibröses Mark mit zahlreichen Osteoklasten auf. Dieses Bild, das einer lokalisierten Osteodystrophia fibrosa gleicht, bezeichnen wir als Stadium der Kompensation, da nun anscheinend der Organismus imstande ist, die Vitamin D_3-Wirkung zu paralysieren und sich dabei anschickt, den osteosklerotischen, im Überschuß gebildeten Knochen durch Resorption abzubauen, wieder zu wachsen und damit das Knochenbild zu normalisieren.

Wie wir einleitend erwähnten, ist es nicht möglich, diese Veränderungen in ihrem Ablauf in jeder Phase mittels histologischer Methoden quantitativ zu differenzieren. Wir können aber in der letzten Phase (5. Versuchswoche) Unterschiede in der Ausbildung des Stadiums der Kompensation, das wir besonders an den Versuchsgruppen mit niedriger Vitamin D_3-Dosierung studierten, zwischen den einzelnen Gruppen herausarbeiten.

Wir zählten die Häufigkeit dieses *histologischen Merkmals der Kompensation* in der fünften Versuchswoche aus und kommen zu folgendem Ergebnis:

Versuchsgruppe	Zahl der Ratten	Kompensationsstadium		
		voll ausgeprägt	angedeutet	in Spuren
5 N	16	7	6	3
5 B	13	3	8	2
10 N	12	—	6	1
10 B	12	1	—	1
DEK	9	5	3	—

Bei der Betrachtung obiger Zusammenstellung fällt auf, wenn wir von den parathyreopriven Tieren (DEK) absehen, daß die 5 N-Gruppe dieses histologische Merkmal weit häufiger und viel ausgeprägter aufweist. Beim Vergleich der 5 N-Gruppe mit der 5 B-Gruppe ist prozentual zur Zahl der überlebenden Ratten das voll ausgeprägte Stadium der Kompensation in der 5 N-Gruppe am meisten vertreten. Dieser Unterschied zwischen den mit und ohne Bolus alba gefütterten Ratten wird noch deutlicher beim Vergleich der 10 N-Gruppe mit der 10 B-Gruppe. Die letalen Ausfälle sind hier nur z. T. erfaßt. Vergleicht man hier schon das Ergebnis dieser Auszählung mit der oben angeführten Mortalität bei den einzelnen Versuchsgruppen, so findet man eine Übereinstimmung der Ergebnisse zumindest insoweit, daß *zwischen den mit Bolus gefütterten und den gleich hoch mit Vitamin D_3 dosierten Ratten Unterschiede bestehen, die eine stärkere Schädigung der mit Bolus gefütterten Tiere andeuten.*

In der Endphase des Versuches wird darüber hinaus vor allem bei den 10 B-Ratten das histologische Bild weitgehend von einer ausgeprägten

Osteosklerose der Metaphyse beherrscht. Die Knochenpartien erscheinen hier besonders kalkreich und dicht. Aber schon bei der 10 N-Gruppe ist im Durchschnitt eine gewisse Auflockerung der Spongiosa zu bemerken. Die Kalkablagerung erscheint nicht mehr so intensiv. Offensichtlich und deutlich gegenüber diesen beiden Gruppen ist der Knochen der 5 N- und 5 B-Gruppe aufgelockert und die Kalkdichte vermindert. Das steht im Einklang mit dem verstärkten Auftreten der resorptiven Prozesse in diesen Versuchsgruppen.

Wir haben in Abb. 2 von je 5 etwa gleichgewichtigen männlichen Ratten der Versuchsgruppen 10 N und 10 B die *Röntgenaufnahmen* des distalen Femurkopfes und des proximalen Tibiaendes gegenübergestellt. Das Röntgenpositiv ist etwa dreimal linear vergrößert.

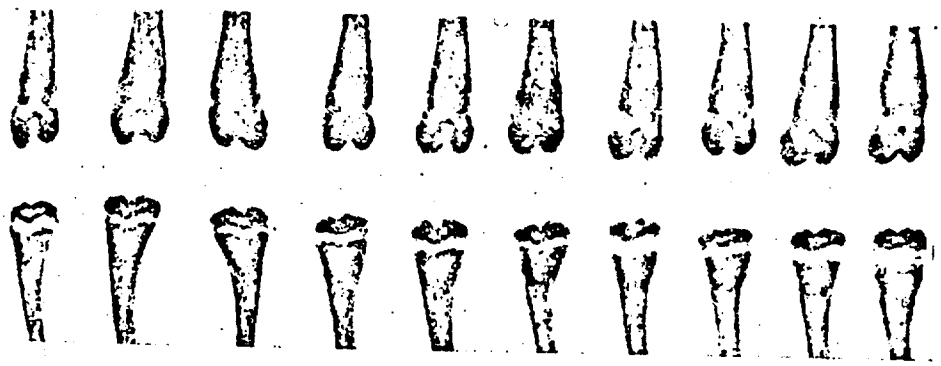

Abb. 2. Röntgenpositiv Tibia und Femur, 5. Versuchswoche
Von links nach rechts: Knochen 1 bis 5 Versuchsgruppe 10 B ♂. Knochen 6 bis 10 Versuchsgruppe 10 N ♂

Da diese Knochen mit einer einzigen Röntgenaufnahme erfaßt wurden, sind wir berechtigt, aus der Schattendichte Rückschlüsse auf den Verkalkungsgrad der Knochen zu ziehen. Nur beim ersten Knochen der 10 B-Gruppe ist eine leichte Aufhellung entsprechend der oben beschriebenen Auflockerung der Spongiosa der Metaphyse zu bemerken, während bei den Knochen aus der 10 N-Gruppe (von links 6 bis 10) diese Aufhellung der Metaphyse bei allen Tieren mehr oder weniger stark zu erkennen ist. Auch ist die Zone der primären Verkalkung (Richtungsbälkchen) bei der Tibia der 10 N-Gruppe deutlich verbreitert, während sie bei der Tibia der 10 B-Gruppe (von links 1 bis 5) relativ schmal und scharf distalwärts gegen hellere Spongiosa abgesetzt ist. Ähnlich liegen die Verhältnisse bei den ♀ Ratten dieser Versuchsgruppen. Es würde zu weit führen, auch die röntgenologisch erfaßbaren Unterschiede zwischen den Versuchsgruppen mit niederer Dosierung des Vitamin D_3 hier aufzuführen, die sinngemäß die obigen Befunde des Unterschieds zwischen den beiden zur Diskussion stehenden Versuchsgruppen bestätigen.

Da das Kompensationsstadium den Ausgleich und die Gewöhnung des Organismus an die Vitamin D_3-Vergiftung anzeigt, bzw. den Rückschluß auf ihre Intensität zuläßt, kann man folgern, daß die Ratten unter den

5 N-Bedingungen noch am ehesten mit der Hypervitaminose fertig werden und die Wirkung des Vitamin D_3 allmählich kompensieren können, während bei 5 B-Ratten dieser Ausgleich auch deutlich, aber doch nicht so wirksam wird. Unter den Bedingungen der 5 B-Gruppe muß also die Vitamin D_3-Wirkung stärker und anhaltender sein. Entsprechend dieser Beurteilung findet man bei doppelt intensiver Vergiftung zu Versuchsbeginn (10 N- und 10 B-Gruppe) histologische Veränderungen im Sinne einer Kompensation gegen Versuchsende nur angedeutet, wobei die 10 B-Tiere sich noch durch eine stärkere Belastung auszeichnen. Es besteht demnach im Gesamtbild eine abgestufte Wirkung, die bei der 5 N-Gruppe beginnt und bei der 10 B-Gruppe endet.

Entsprechendes sieht man bei der chemisch-analytischen Untersuchung der langen Knochen. Wir untersuchten Tibia und Femur auf fettfreie Trockensubstanz, Asche, Calcium und Phosphor. Hier beschränken wir uns auf die Wiedergabe der *Untersuchungsergebnisse des Aschegehaltes* dieser Knochen. Aus den Untersuchungen auf Calcium und Phosphor konnten wir keine instruktiveren Beziehungen ableiten, auch brachte das Ca : P-Verhältnis der Knochen keine differenzierbaren Unterschiede, die zunächst zur weiteren Klärung der Fragestellung beitragen könnten.

Da es bei der Vitamin D_3-Hypervitaminose zu einer Wachstumshemmung kommt, suchten wir nach einer Möglichkeit, diese auch bei der Betrachtung des Mineralstoffgehaltes in Beziehung zu setzen. Die Körpergewichtsentwicklung der Ratten erschien uns dafür nicht der geeignete Maßstab zu sein, da die Tiere in der Versuchsfolge z. T. ihr Anfangsgewicht unterschreiten, was ein Ausdruck der Substanzeinschmelzung und nicht des Wachstumsstillstandes sein kann. Wir wählten deshalb als Vergleichsmaßstab für das Wachstum die Tibialänge, da nicht anzunehmen ist, daß der Knochen in der Lage wäre, Substanzänderungen in dem Maße der Schwankungen des Körpergewichts mitzumachen.

Der Knochen vermag sein Volumen mit mehr oder weniger großen Substanzmengen unter Strukturwandlung auszufüllen. Dabei können bei gleich hohem Substanzgehalt, sei es organisches oder anorganisches Material, verschieden hohe Mengen Wasser zum Aufbau herangezogen werden und damit ein verschieden großes *Längenwachstum* erreicht werden. Der Vergleich absoluter Werte von Knochensubstanz bringt demnach keine befiedigenden Ergebnisse für den Grad der Strukturbeschaffenheit in einer bestimmten Entwicklung und das Wachstum entwicklungsfähiger Knochen. Die Beziehung der anorganischen Substanz auf die fettfreie Gesamttrockensubstanz des Knochens gibt mehr Einblicke in den Strukturumbau desselben ohne den Faktor Wachstum genügend zu berücksichtigen.

Dem Längenwachstum entsprechend nimmt beim normalen Knochen der *Aschegehalt* zu. Beim jungen, entwicklungsfähigen Knochen, mit dem wir es hier zu tun haben, folgt die Entwicklung des Aschegehaltes der Knochen tendenzmäßig weitgehend der Körpergewichtsentwicklung der Ratten. Wenn wir nach dem weiter oben Gesagten es bei der Vitamin D_3-Hypervitaminose mit wechselnden, histologisch erfaßbaren Strukturänderungen

des Knochens zu tun haben und gleichzeitig im Vergleich mit den Kontrollen mehr oder weniger starke Wachstumshemmungen auftreten, so muß dieses Wachstum durch einen besonderen Faktor beim Vergleich chemisch-analytisch bestimmbarer Knochenbestandteile herangezogen werden.

Es erschien interessant, die Länge der Tibia des Einzeltieres zu ihrem Aschegehalt in Beziehung zu setzen. Dabei wurde die Länge der Tibia in $^1/_{10}$ mm von denselben Punkten des Condylus fibularis zum Malleolus tibiae der einzelnen Knochen gemessen und diese Länge bei der Berechnung des Verhältnisses: „% Asche in der ursprünglichen Substanz zu Länge der Tibia" in cm eingesetzt. Abb. 3 zeigt die Mittelwerte dieser Beziehung graphisch für die Versuchsfolge und die einzelnen Versuchsgruppen dargestellt.

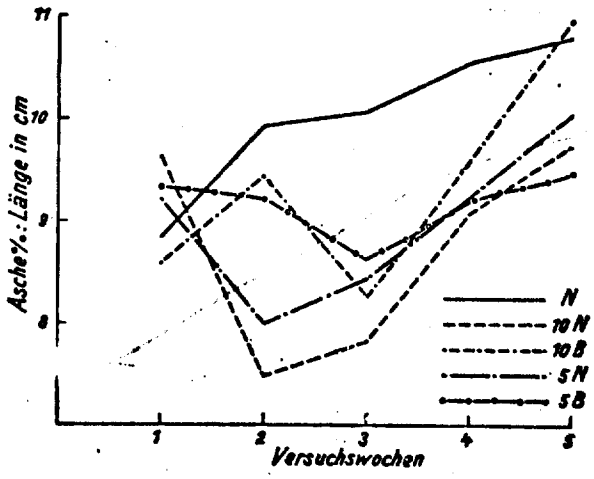

Abb. 3. Tibia: Verhältnis Asche % in der ursprünglichen Substanz zu Tibialänge in cm

Die *chemisch-analytischen Werte der Tibia* stehen in einem korrespondierenden Verhältnis zu denen des Femurs. Tendenzmäßig besteht kein Unterschied zwischen diesen beiden Knochen in bezug auf ihre Veränderungen bei der Vitamin D_3-Hypervitaminose. Wir haben nun als Beispiel für den Zusammenhang analytischer Werte mit dem histologischen Bild den prozentualen Aschegehalt in der Trockensubstanz des Femur für die Aufklärung der Unterschiede zwischen den Versuchsgruppen herangezogen. Die Tibia verwandten wir zur Längenbestimmung, da sie sich für diesen Zweck besser als der Femur eignet. In Abb. 4 sind die Mittelwerte dieses Verhältnisses für die Versuchsfolge und die einzelnen Versuchsgruppen graphisch zusammengestellt.

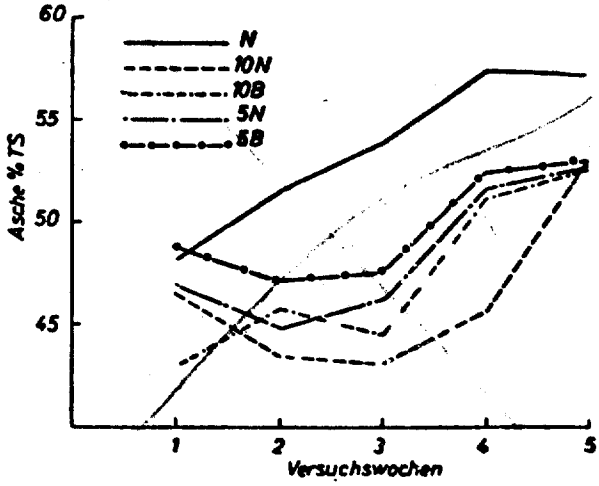

Abb. 4. Femur: % Asche in der fettfreien Trockensubstanz (TS)

Nehmen wir zunächst zur Erörterung des unterschiedlichen Verhaltens der Versuchsgruppen in der *Knochenausbildung* die zweite Versuchswoche

heraus. Hier zeigt sich gemäß dem eingangs erörterten Phasenverlauf der Knochenveränderungen bei der Vitamin D_3-Hypervitaminose bei den mit Bolus alba gefütterten Rattengruppen 5 B und 10 B ein gegenüber den vergleichbaren Versuchsgruppen 5 N und 10 N ausgeprägt erhöhter Asche-Tibialängenwert, der einerseits durch verstärkte Wachstumshemmung des Knochens (Abb. 3) und andererseits durch eine erhöhte Mineralstoffeinlagerung zustande kommt. Letzteres geht aus Abb. 4 hervor, in der wir den *Aschegehalt der fettfreien Trockensubstanz des Femur* dargestellt haben. Wir müssen hier darauf hinweisen, daß histologisch in diesem Versuchsstadium das Bild der Osteosklerose bei allen vergleichbaren Versuchsgruppen besteht. Demnach sind sowohl Wachstumshemmung als auch Verkalkungsgrad bei den B-Gruppen in diesem Stadium ausgeprägter als bei den vergleichbaren N-Gruppen. Diese histologisch bereits besprochenen Unterschiede zwischen den zur Diskussion stehenden Versuchsgruppen finden hier ihre quantitative Bestätigung.

Betrachten wir die Verhältnisse in der fünften Versuchswoche, also zu Versuchende, so finden wir die histologischen Befunde auch hier quantitativ bestätigt. Während gemäß Abb. 4 kaum noch Unterschiede im prozentualen Aschegehalt der fettfreien Trockensubstanz zwischen den einzelnen Versuchsgruppen zu verzeichnen sind, finden wir diese jedoch ausgeprägt bei Betrachtung des Asche-Tibialängenverhältnisses (Abb. 3). Hier zeigt sich besonders bei der 10 B-Gruppe die starke Wachstumshemmung im Vergleich mit den übrigen Versuchsgruppen. Eine Differenzierung der niedrig dosierten Versuchsgruppen ist in dieser Phase auch möglich, wenn man in Betracht zieht, daß die Vitamin A-Gabe in der dritten Versuchswoche einen Einfluß auf die Tibialänge haben könnte. Wir werden diese Möglichkeit weiter unten diskutieren.

Es bedarf noch des Hinweises, daß hier ein Vergleich mit den Normaltieren nicht angebracht ist, wenn auch die B-Gruppen, besonders die 10 B-Gruppe in der fünften Versuchswoche und in den ersten beiden Versuchswochen eine ähnliche Tendenz des Mineralisierungsvorganges (Abb. 3 und 4) wie die Normalgruppe zeigt. Diese Gruppen sind allein deshalb nicht vergleichbar, weil es sich hier um einen histologisch grundsätzlich andersartigen Prozeß der Verknöcherung handelt.

Aus den bisher besprochenen Knochenveränderungen geht eindeutig hervor, daß die Vitamin D_3-Hypervitaminose in ihren Auswirkungen durch Bolus alba-Fütterung alimentär beeinflußbar ist.

Die Veränderungen der Epithelkörperchen (EK)

Die Auswertung der EK erfolgte in zweierlei Weise. Die erste Form ging von den eigenen früheren experimentellen Erfahrungen aus, daß Nebenschilddrüsen sich bei anhaltender funktioneller Belastung vergrößern und die Volumenzunahme parallel mit der Hyperfunktion und ihren Auswirkungen geht[9]. Aus dieser Größenveränderung kann man also Rückschlüsse auf den Funktionszustand der Drüsen ziehen.

Für diese Form der Auswertung wurden die EK bzw. die Halsorgane der Versuchstiere nach Einbettung in der üblichen Weise in 5 μ-Serien aufgeschnitten und jeder 5. Schnitt aufgezogen, gefärbt, auf das 100fache vergrößert und der Umfang der EK mittels eines Zeichenapparates auf gleichmäßig dicke Pappe projiziert, ausgezeichnet und ausgeschnitten. Die aus einer Serie des einzelnen EK gewonnenen Pappblättchen wurden zusammen gewogen. So erhält man zwar nicht absolute, sondern *„relative" Gewichte*, die wir mit den Werten der Normaltiere in Vergleich setzen können. Das Ergebnis ist in folgender Tabelle und in Abb. 5 zusammengestellt.

Versuchswoche	Versuchsgruppe				
	N	5 N	5 B	10 N	10 B
1	2,73 ± 0,20	2,67 ± 0,4	2,27 ± 0,06	1,90 ± 0.19	2,70 ± 0,49
2	3,22 ± 0,30	2,56 ± 0,44	3,06 ± 0,37	2,14 ± 0,38	2,10 ± 0,28
3	5,39 ± —	2,13 ± 0,09	2,48 ± 0,31	2,02 ± 0,20	1,71 ± 0,14
4	5,28 ± 0,28	2,55 ± 0,4	2,63 ± 0,27	2,07 ± 0,17	2,52 ± 0,23
5♂	5,48 ± 0,28	2,38 ± 0,18	2,26 ± 0,16	1,94 ± 0,14	1,62 ± 0,08
5♀	4,49 ± 0,34	2,49 ± 0,11	2,49 ± 0,25	2,18 ± 0,2	1,92 ± 0,14

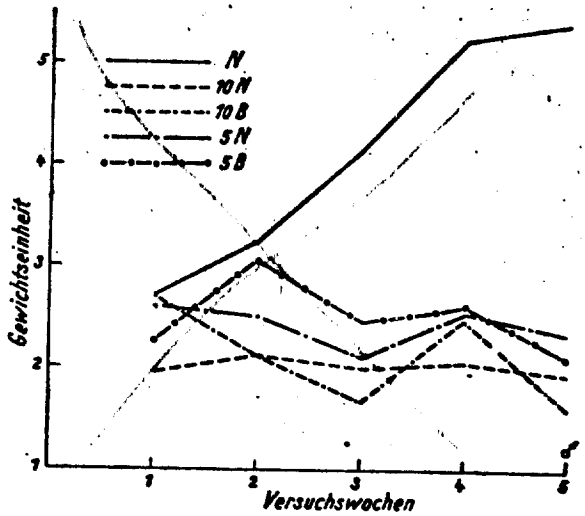

Abb. 5. „Absolutes" Gewicht der Epithelkörperchen

Aus Abb. 5 ist zu entnehmen, daß bei den Kontrolltieren das *Gewicht der EK* anfänglich gleichmäßig zunimmt. Nach der vierten Versuchswoche, nach dem die Normaltiere bereits ein Gewicht von 150 g erreicht haben, steigt das Gewicht der EK nur noch wenig. Die weiblichen Tiere sind in der fünften Versuchswoche durch ein geringeres EK-Gewicht ausgezeichnet. In den mit Vitamin D_2 vergifteten Versuchsgruppen verlaufen die Gewichtskurven ziemlich gleichmäßig parallel der Abszisse im Gesamtbild, wenn man von einigen im Vergleich zum Kurvenverlauf bei den Kontrolltieren geringfügigen Schwankungen absieht. Doch ergeben sich bereits nach der zweiten Versuchswoche deutliche Unterschiede dahingehend, daß die stärker vergifteten Ratten eine höhere Reduktion ihres EK-Volumens erleiden. In der dritten Versuchswoche erreicht das EK-Gewicht bei den 10 B-Ratten ein Minimum, das unter dem aller übrigen Gruppen liegt und anzeigt, daß diese Tiere übereinstimmend mit unseren Ergebnissen am Knochen am stärksten geschädigt

sind. In der vierten Versuchswoche macht sich bei allen Tiergruppen ein leichter Gewichtsanstieg bemerkbar, der bei den 10 B-Ratten besonders deutlich wird. In dieser Zeit erhielten die Tiere, da ihr Körpergewicht allgemein zu stark abfiel und ein vorzeitiger Verlust zu befürchten war, 2 Tage lang Vitamin A als Vitamin D_3-Gegengabe, wonach sie sich vorübergehend erholten. Diese Remission kommt in der Volumenzunahme der EK zum Ausdruck.

Am Ende des Versuches ist dann wieder die Abstufung zu sehen, die wir auch am Knochen feststellten. Die 5 N-Ratten liegen mit ihrem EK-Gewicht noch am höchsten. Es folgen mit stetig geringer werdendem EK-Gewicht die 5 B, die 10 N- und dann die 10 B-Tiere Die Intensität der Vitamin D_3-Vergiftung nimmt also in dieser Reihenfolge zu und macht sich in der EK-Verkleinerung bemerkbar.

Der zweiten Form der Auswertung der EK liegt die Erkenntnis zugrunde, wie wir schon einleitend betonten, daß die *Kerngröße* eines parenchymatösen Organes etwas über den Funktionszustand der Zelle aussagt, wobei die Zunahme des Kernvolumens eine erhöhte Aktivität anzeigt und umgekehrt.

In neuester Zeit wendet man dieses Verfahren sehr häufig zur Beurteilung innersekretorischer Drüsen an. An den EK wurde die Methode von Engfeld und von Hanssler erprobt. Engfeld untersuchte vor allem die Beziehungen der Nebenschilddrüsen zur Hypophyse und Hanssler zu Vitamin D_2 und D_3. Die Ergebnisse des letzteren sind vor kurzem erschienen, nachdem unsere Experimente schon durchgeführt und ausgewertet waren. Er findet bei täglicher Verabreichung von etwa 30 γ Vitamin D_2 oder D_3 an Ratten von 50 bis 70 g nach 6 bis 13 Tagen eine Ruhigstellung der EK, die sich in einer Verkleinerung der Kerne ausdrückt. Bemerkenswert erscheint uns, daß die Kontrolltiere wie die Versuchstiere dieses Autors nach den Gewichtstabellen in dieser Zeit nicht an Gewicht zunahmen. Auch Engfeld konnte in seinen sorgfältigen Untersuchungen durch geeignete Bedingungen die Funktion der EK hemmen oder steigern und eine entsprechende Vergrößerung oder Verkleinerung der Kerne sehen.

Die Bestimmung der Kerngröße kann auf zweierlei Art erfolgen. Man mißt entweder den Durchmesser und berechnet daraus das Volumen oder bestimmt die im Schnitt aufscheinenden optisch größten Kernflächen durch Planimetrieren. Die erste Methode kann nur dann angewandt werden, wenn die Kerne gleichmäßig rund sind. Nehmen sie ovale Gestalt an, muß man zwei Durchmesser bestimmen und daraus den Inhalt des Kernes berechnen. Das würde für das EK der Ratte zutreffen, bei der die Kerne vorwiegend länglich-oval sind. Bei genauer Betrachtung aber selbst von Organkernen, die auf den ersten Blick rund erscheinen, zeigt sich, daß sie keineswegs eine gleichmäßig runde Gestalt haben. Man wird fast bei jeder Kernmessung vor das Problem gestellt, welchen Durchmesser man anlegt. Die Fehlermöglichkeit ist dabei erheblich und wird noch durch die subjektive Einstellung des Untersuchers erhöht, dieser Fehler dann durch die Volumenberechnung potenziert. Diese Methode wird aber heute vielfach bei der Auswertung der Nebennieren unter der falschen Voraussetzung, daß die Kerne

der Rinde rund sind, angewandt und auch von HANSSLER für die EK, indem er zwei Durchmesser bestimmt, benutzt.

Wir entschieden uns nach sorgfältiger Prüfung der Methoden für das Planimetrieren, zumal die Untersuchungen von ENGFELD mit dieser Methode durchgeführt uns einen überzeugenden Eindruck machten. Auch dieser Auswertung haften allerdings Mängel an, zumal nicht das Volumen, sondern nur die Fläche gemessen wird und man durch die Schnittführung des histologischen Präparates nicht in jedem Fall den optisch größten Durchmesser des Kernes erfaßt. Man reduziert aber die subjektiven und objektiven Fehlermöglichkeiten auf ein erträgliches Mindestmaß.

Im einzelnen werden die 5 μ dicken Schnitte der EK 1300fach vergrößert, auf dünne Pappe der Umfang ausgezeichnet und dann planimetriert. Da die Flächen klein sind, arbeiten wir mit einem kurzen Planimeterarm (40 mm) und erhalten dadurch größere Ausschläge. Der Kern wird dreimal umfahren, der Gesamtwert gedrittelt. Der Fehler der Methode beträgt 2%, ist also unerheblich.

Aus jeder Versuchsgruppe wurden 2 Tiere jeweils mit dem höchsten und niedrigsten Körpergewicht ausgewertet und je Tier 200 Kerne (im ganzen 12000) ausgemessen. Die erhaltenen Zahlen stellen nicht absolute, sondern direkt abgelesene Relativwerte dar. Die ausgewerteten 2 Tiere einer Versuchswoche, also jeweils 400 Kerne, werden zusammengefaßt und die Häufigkeitsverteilung der Größenklassen der Kerne in ein Koordinatensystem eingetragen.

Wir verzichten absichtlich auf eine logarithmische und prozentuale Aufstellung der Verteilungskurven, da durch diese Art der Darstellung wissenschaftlich nicht mehr erreicht wird und in unserem Material manche Unterschiede nicht so deutlich zum Ausdruck kommen.

Abb. 6 bringt die *Kernkurven der Normaltiere* für die gesamte Versuchsfolge. An ihnen fällt auf, daß sich mit zunehmendem Wachstum der Ratten

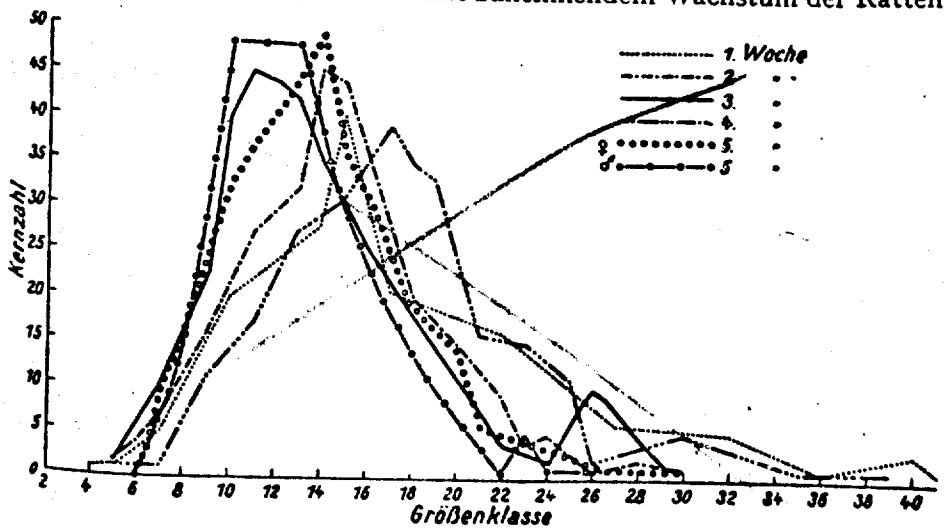

 Abb. 6. Kerngröße der Epithelkörperchen: Normaltiere

die Gipfel der Kurven nach links verschieben, die Kerne also kleiner werden. In der vierten Versuchswoche springt die Kurve jedoch nochmal über den Ausgangswert zurück. Die Verschiebungen sind nach der statistischen Berechnung signifikant. Die mit dem fortschreitenden Wachstum abnehmende Kerngröße der EK, dürfte wohl damit zusammenhängen, daß die Knochenentwicklung allmählich an Intensität und damit der Umsatz an Ca abnimmt und damit an die EK geringere Anforderungen gestellt werden, deren Funktion somit herabgesetzt wird. Ähnliche Befunde konnte auch Engfeld an wachsenden Ratten erheben. Auffallend ist die Unterbrechung der Kerngrößenabnahme in der vierten Versuchswoche, vielmehr zeigt sich hier eine beträchtliche Vergrößerung, so daß der Kurvengipfel um sechs Größenklassen nach rechts verschoben wird. Wir müssen dieses Verhalten zunächst auf die Vitamin A-Gabe zurückführen, es aber offen lassen, wie diese Wirkung im einzelnen zu erklären ist. Allein aus dem Befund wäre hier zu folgern, daß die EK zu einer beträchtlichen Funktionssteigerung veranlaßt werden, der Ca : P-Stoffwechsel in dem Sinne beeinflußt würde, daß eine vermehrte Parathormonproduktion notwendig ist. Wir werden weiter unten darauf zurückkommen.

Der Hinweis auf die *Schwankung der Kerngröße in den Wachstumsphasen* erscheint uns besonders wichtig, um von vornherein zum Ausdruck zu bringen, welche Faktoren normalerweise die Kerngröße von Ratten-EK beeinflussen und daß kleinere Unterschiede mit größter Vorsicht zu beurteilen sind, auch wenn durch die große Zahl der Messungen die statistische Berechnung der Unterschiede signifikant erscheint.

In Abb. 7 a u. b haben wir die *Kernkurven der Versuchsgruppen* 5 N und 5 B zusammengestellt, also derjenigen Gruppen, die anfänglich die niedere Dosis Vitamin D_3 erhielten. Wir geben zum Vergleich eine Durchschnittskurve der Normaltiere bei und lassen der besseren Übersicht halber die weiblichen Tiere der fünften Versuchswoche fort, um das Bild nicht unnötig zu verwirren.

Man sieht im Vergleich mit den Normaltieren bei der 5 N-Gruppe mit der ersten Woche beginnend die Kurven stetig steiler werdend und die Gipfel der Kurven noch links rückend, bis nach der dritten Versuchswoche diese Verschiebung des Kuvenbildes rückläufig wird. In der vierten Versuchswoche rückt analog dem Befund bei den Normaltieren die Kernkurve um eine Größenklasse nach rechts, was bei allen Ratten zum Ausdruck kommt und sich auch in der vorübergehenden Gesamtgewichtszunahme der EK in dieser Phase anzeigte.

Die Kerne der EK werden also nach dem Kurvenbild bei zunehmender Vergiftung mit Vitamin D_3 kleiner und in ihrer Gestalt uniform, was wir später in den histologischen Bildern deutlich demonstrieren können. Die Gipfel der Kurven gehen von der Größenklasse 14 bis auf 9 und 10 zurück und steigen abschnittsweise bis zur dritten Woche von der Häufigkeitszahl 40 auf den Wert 75 an.

Ähnlich verhalten sich die Kernkurven bei den 5 B-Ratten. Der Unterschied gegenüber den 5 N-Tieren besteht darin, daß die Linksverschiebung

der Kurvenbilder in der Versuchsfolge noch um eine Größenklasse weitergeht, den Wert 8 erreicht und die Kurvenhöhe die Häufigkeitszahl 80 überschreitet. Demnach ist in dieser Gruppe die Kernverkleinerung und die Uniformierung noch stärker ausgeprägt als in der Versuchsgruppe 5 N. Wir sehen darin wieder eine Bestätigung der verstärkten toxischen Vitamin D_3-Wirkung bei Bolus alba-Fütterung.

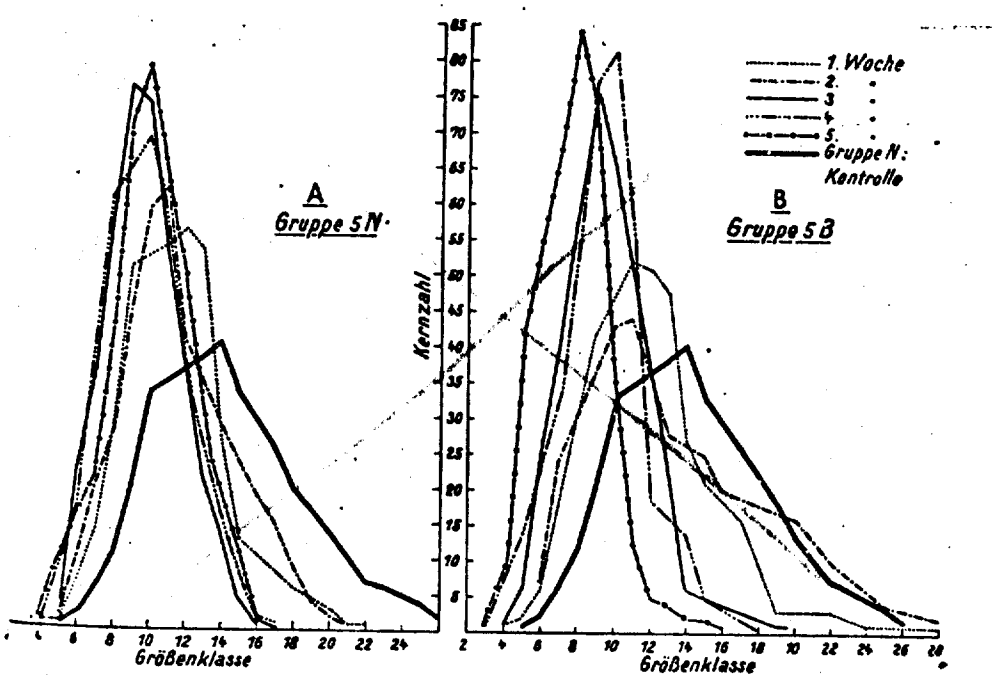

Abb. 7. Kerngröße der Epithelkörperchen. a) Gruppe 5 N, b) Gruppe 5 B

Auch in Abb. 8, die das *Verhalten der EK-Kerne der Versuchsgruppen 10 N und 10 B* veranschaulicht, zeigt sich deutlich die Linksverschiebung und Erhöhung der Kernkurven im Versuchsablauf. Gegenüber den vorhergehend besprochenen Versuchsreihen erfolgt sie aber nicht von Woche zu Woche, sondern ist mit einem Sprung von der ersten zur zweiten Woche völlig ausgeprägt.

Im einzelnen erscheint bemerkenswert, daß die Kernkurven der 10 N-Ratten sich nicht so weit nach links verlagern wie die der 5 B-Tiere. Ihre Gipfel liegen bei den Größenklassen 9 und 10, sie gleichen demnach in ihrer Ausbildung weit mehr denen der 5 N-Gruppe, während andererseits die Kurven der 10 B-Gruppe in Linksverschiebung und Kurvenhöhe eher mit den Kurven der 5 B-Tiere übereinstimmen. Bei dieser Auswertung macht sich also nicht so sehr der Unterschied der Dosierung als der Einfluß des Bolus alba bemerkbar.

Durch Vitamin D_3-Vergiftung verkleinern sich also die Kerne der EK wahrscheinlich als Ausdruck der Funktionshemmung, wobei die Dosierung

des Vitamins D_3 in den angegebenen Grenzen keinen deutlichen Wirkungsunterschied, der Zusatz von Bolus alba zur Nahrung dagegen eine signifikante Differenz im Sinne einer verstärkten Hypervitaminose hervorruft.

Man könnte nun mit dem Ergebnis der Auswertung der EK zufrieden sein, zumal sie in bester Übereinstimmung mit den Befunden an den Knochen steht und doch haben wir uns gefragt, ob es sich bei der Verkleinerung des

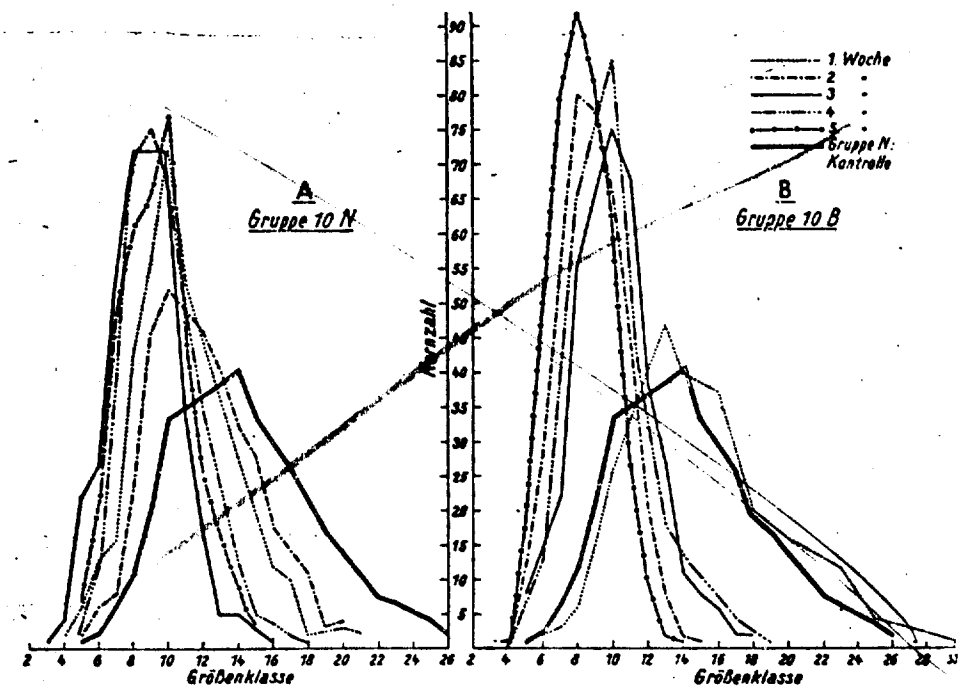

Abb. 8. Kerngröße der Epithelkörperchen. a) Gruppe 10 N, b) Gruppe 10 B

Gesamtvolumens der EK und ihrer Kerne um den Ausdruck einer tatsächlichen funktionellen Hemmung oder nur um eine Reduktion durch allgemeine Schädigung des Organismus handelt.

Um diese Frage weiterzuverfolgen, wurde die Kerngröße zum Gesamtvolumen der EK in Beziehung gesetzt und damit in gewisser Weise eine *Kern-Plasmarelation* erreicht. Erst eine solche Auswertung dürfte sich einer *umfassenden* Bewertung am meisten nähern, da doch wohl die Kerne zusammen mit dem Cytoplasma an der Funktion der Zelle beteiligt sind. Man hat sich in diesem Sinne verschiedentlich um die Erfassung der Kern-Plasmarelation bemüht. Es ist methodisch ein schwieriges Problem, das bis heute nicht befriedigend gelöst ist. Wir wollen auf die Einzelheiten nicht näher eingehen. Auch die vorliegende Art der Beziehung ist nicht einwandfrei in ihrer Zusammenstellung. Sie erscheint uns aber doch instruktiv und zusammen mit dem histologischen Bild das Wesentliche unserer Frage zu berühren.

In Abb. 9 ist diese *Kern-Plasmabeziehung kurvenmäßig* für die Versuchsfolge aufgezeichnet. Wir möchten uns aber bei der Besprechung der Ergebnisse auf die Normaltiere und die 5 B- und 10 B-Gruppe beschränken, um nicht die Auswertung zu komplizieren.

Bei den Normaltieren fällt das Kern-Plasmaverhältnis mit zunehmendem Wachstum rasch ab, um ab der dritten Versuchswoche mit kleinen Schwankungen auf derselben Höhe zu bleiben. Die 5 B-Ratten zeigen einen kontinuierlichen und geringeren Abfall der Relation. Bei den 10 B-Ratten bleibt die Relation etwa auf derselben Höhe, zeigt aber stark wechselnde Tendenz, die gegenläufig in auffallender Übereinstimmung mit dem Asche-Tibialängenverhältnis (Abb. 3) steht. Dieser Wechsel kann durch die Größenänderung der Kerne oder durch Volumenschwankung des Protoplasma bedingt sein, ebenso wie das Asche-Tibialängenverhältnis die Funktion der Mineralisierung und das Wachstum des Knochens widerspiegelt. Daß beide Teile an dieser Schwankung beteiligt sind, geht aus dem Vergleich absoluter Werte hervor.

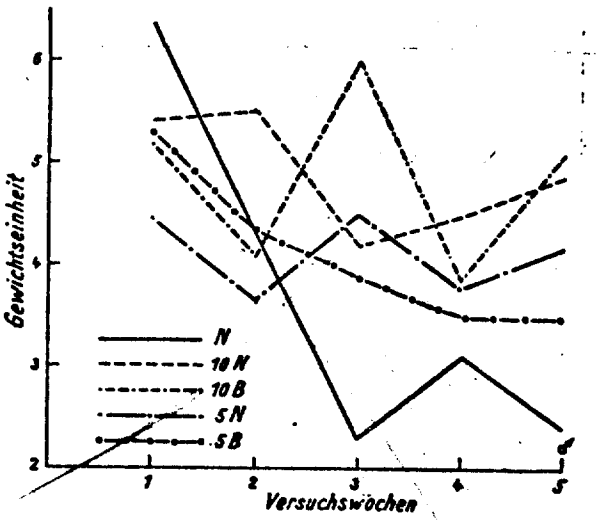

Abb. 9
Verhältnis der Kerne zum Gesamtvolumen der Epithelkörperchen

Es ist nun interessant, diese Schwankungen am *histologischen Bild der EK* zu verfolgen, das deutlich den Kurvenverlauf (Abb. 9) widerspiegelt. Wir wählen dazu die eben genannten drei Versuchsgruppen aus und stellen in Abb. 10 das histologische Bild der EK in der ersten, dritten, vierten und fünften Woche gegenüber.

In der Reihe der Normaltiere (Abb. 10) sieht man deutlich, daß die Kerne in der dritten Versuchswoche kleiner als in der ersten Versuchswoche sind, dann in der vierten Woche leicht an Größe zunehmen, um zum Versuchsende wieder kleiner zu werden. Der Verlauf der Kern-Plasmarelation (Abb. 9) scheint demnach im wesentlichen durch das Verhalten der Kerne bedingt. In der Versuchsgruppe 5 B (Abb. 10, mittlere Reihe) sind die Kerne im Vergleich mit denen der Normaltiere erheblich in ihrem Volumen reduziert und statt oval und länglich jetzt weitgehend abgerundet, dicht stehend und durch ein gleichbleibendes Verhältnis zum Cytoplasma entsprechend den in Abb. 9 wiedergegebenen Kurvenverlauf charakterisiert.

Bei der 10 B-Gruppe (Abb. 10, untere Reihe) kommt der Wechsel der Kern-Plasmarelation eindringlich zum Ausdruck. Das erste Bild entspricht noch dem der darüberliegenden Reihe der 5 B-Gruppe. In der dritten Ver-

suchswoche sind die Kerne abgerundet, dicht stehend und das Cytoplasma ist weitgehend geschwunden. Die Kern-Plasmarelation (Abb. 9) steigt entsprechend der Kurve zugunsten der Kerne steil an, obgleich sie absolut gesehen auch beträchtlich an Größe verlieren. Im weiteren Versuchsverlauf nimmt das Cytoplasma in dieser Versuchsgruppe wie das Kernvolumen er-

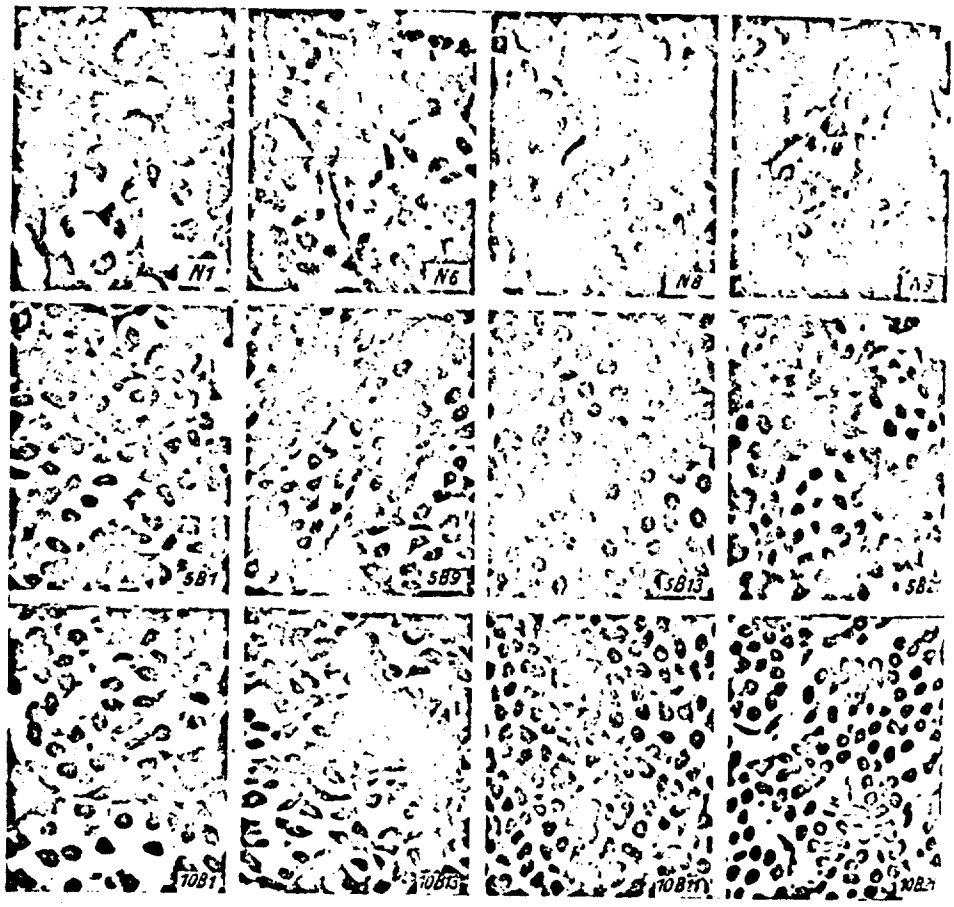

Abb. 10. Histologisches Bild der Epithelkörperchen

Obere Reihe: Versuchsgruppe N: Normaltiere. Mittlere Reihe: Versuchsgruppe 5 B. Untere Reihe: Versuchsgruppe 10 B. Von links nach rechts: 1., 3., 4. und 5. Versuchswoche

heblich an Masse zu, um in der fünften Versuchswoche wieder denselben Zustand wie in der dritten Versuchswoche zu zeigen. Dieses wechselnde Verhalten, bzw. die offensichtliche funktionelle Erholung in der vierten Versuchswoche dürfte bei der 10 B-Gruppe auf die schon oben erwähnte Zufütterung von Vitamin A und die Entlastung durch zweitägiges Aussetzen der Vitamin D_3-Applikation zurückzuführen sein.

Es liegt auf der Hand, daß erst diese Beobachtungsweise einen umfassenderen Einblick in histologisch funktionelle Änderungen der EK vermittelt.

die Kurven allein zwar einen groben Überblick geben, aber nur für sich betrachtet nicht die tatsächlichen Verhältnisse erkennen lassen. Auf unser vorliegendes Thema bezogen sichert diese Auswertung voll und ganz das Ergebnis der bisherigen Befunde.

Der Zusammenhang der Veränderungen innersekretorischer Drüsen mit den Vitamin A- und D-Gaben

Die Symptome der Vitamin D-Hypervitaminose werden durch Vitamin A[18, 26, 28, 28, 39, 40], Vitamin B-Komplex[1, 22, 24] und z. T. noch unbekannte Fettbegleitstoffe[6, 7] beeinflußt und gemindert. Diese experimentellen Erfahrungen sind Tatsachen, weitgehend unbekannt ist aber noch, ob diese Vitamin D-Gegengaben bei der besprochenen Hypervitaminose lediglich symptomatischer Natur für Folgeerscheinungen der Vitamin D_3-Vergiftung sind oder aber in einem grundsätzlichen Zusammenhang mit der spezifischen Vitamin D_3-Wirkung auf innersekretorische Drüsen und besonders deren Beeinflussung des Mineralstoffwechsels stehen. Aus der Beantwortung dieser Frage könnte man den physiologischen Wirkungsmechanismus von Bolus alba bei dieser alimentären Beeinflussung der Vitamin D_3-Hypervitaminose näher spezifizieren. Die obigen Befunde zeigten diesen Einfluß des Bolus alba deutlich.

In früheren Versuchen war bei normaler Fütterung und Dosierung der Vitamine der *adsorptive Charakter von Bolus alba im Darm nicht nachzuweisen.* Wir konnten z. B. im Wachstumstest bei geringen Vitamin A-Gaben einen alimentären Einfluß dieses extramicellaren Adsorbens nicht erkennen, auch scheiterten Versuche mit geringen Vitamin D-Gaben. *Erst unter den Bedingungen der Vitamin D_3-Hypervitaminose* zeigte sich bei erwachsenen Ratten ein alimentärer Einfluß von Bolus alba[4], der jedoch unter Beachtung der Zusammensetzung der Diät nicht dem adsorptiven Charakter dieses Stoffes entsprach. Da diese Versuche sowohl mit als auch ohne Vitamin A-Zufütterung dasselbe Ergebnis zeitigten, die verabreichten Vitamin A-Gaben dem Angriff des Adsorbens außerdem entzogen wurden, ist eine Resorptionsförderung des Bolus für Vitamin D_3 anzunehmen.

Eine Wechselwirkung zwischen den Vitaminen ist nach den Arbeiten von SCHEUNERT[33, 34, 35, 36] für den normalen Stoffwechselverlauf auszuschließen. Bei den in diesem Experiment gesetzten toxischen Schädigungen müssen wir unsere Befunde daraufhin untersuchen, welche Veränderungen des Organismus als direkte Auswirkung der Vitamin D_3-Gaben anzusehen sind und welche als Sekundärerscheinungen zu gelten haben. Ohne dieses Gebiet jetzt schon abschließend überschauen zu können, haben wir doch den Versuch gemacht in diesem Zusammenhang wenigstens zwischen Vitamin A- und D-Wirkung zu differenzieren, um unsere Organteste weiter zu sichern.

Weiterhin stehen die verzeichneten Knochenveränderungen sicher auch über den Ca- und P-Stoffwechsel mit der Funktion der innersekretorischen Drüsen im Zusammenhang. Die endokrinologische Forschung befaßt sich

nun vornehmlich mit dem Studium von Ausfallerscheinungen nach Endokrindrüsenektomie. Wir führten demnach in unseren Versuchen auch eine parathyreoprive Versuchsgruppe, um den Einfluß der EK auf die Hypervitaminose des Vitamins D_3 mit zu verfolgen.

Die *Extirpation der EK* wurde in der üblichen, früher vielfach geübten Weise, durchgeführt. Zur Technik sei auf diese Arbeiten verwiesen[10]. Da bei Vigantolvergiftungen keine Tetanie als Zeichen der gelungenen Operation zu erwarten ist, müssen die Halsorgane der Tiere in Serien aufgeschnitten und der Erfolg der Operation durch die mikroskopische Musterung der Serien kontrolliert werden.

Die *histologischen Knochenveränderungen* zeigen, wie aus unserer früheren Arbeit hervorgeht[5], keine grundsätzlichen Unterschiede zu den anderen Versuchsgruppen. Sie verhalten sich vielmehr in dem phasenartigen Ablauf der Knochenprozesse völlig gleich und entsprechen nach der Häufigkeit des Kompensationsvorganges der 5 N-Gruppe. Anscheinend wird das Fehlen des EK-Hormons und seine Wirkung durch die Vergiftung mit Vitamin D_3 weitgehend überdeckt. Vergleicht man jedoch die Häufigkeit des Kompensationsstadiums in der EK-Gruppe mit der anderer Versuchsgruppen, so fällt auf, daß alle außer einem parathyreopriven Tier die resorptiven Abbauvorgänge am Knochen entwickelt und mehr als die Hälfte das ausgeprägte Stadium aufweist. Berücksichtigt man noch das histologische Bild im ganzen, so muß man betonen, daß gerade in dieser Tiergruppe das Bild der Osteodystrophia fibrosa besonders ausgeprägt auftritt, der sklerosierte Knochen am weitesten aufgelockert und sein Kalkgehalt reduziert erscheint. Setzt man die *Länge der Tibia zu ihrem Aschegehalt* in Beziehung, so kommt man beim Vergleich der Versuchsgruppen 5 N und DEK zu einer quantitativen Bestätigung der histologischen Befunde. Als Folge der Operation ist bei den parathyreopriven Ratten die Körpergewichtsentwicklung und damit auch in gewissem Grade das Längenwachstum der Tibia gehemmt. Unabhängig davon durchläuft der Knochen in der DEK-Gruppe die oben beschriebenen histologischen Phasen der Knochenveränderungen. Im Vergleich mit der 5 N-Gruppe erscheint der prozentuale Aschegehalt in der Trockensubstanz der Tibia, wie wir in der vorhergehenden Arbeit verzeichneten[5], bei den parathyreopriven Ratten zu Versuchsbeginn etwas höher. Dies kommt auch (Abb. 11) in dem Verhältnis Asche-Tibialänge zum Ausdruck, indem der Wert dieses Verhältnisses zu Versuchsbeginn den der 5 N-Gruppe etwas übertrifft.

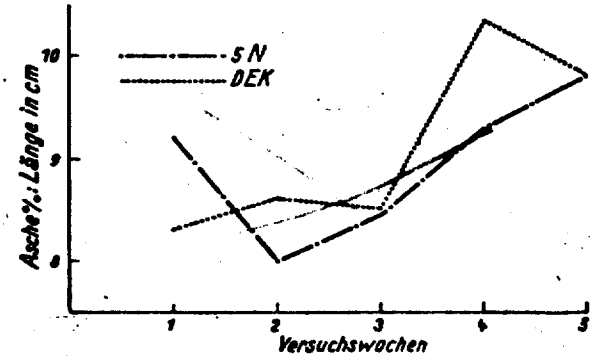

Abb. 11. Tibia: Verhältnis Asche % in der ursprünglichen Substanz zu Tibialänge in cm

Wie aus Abb. 11 jedoch deutlich hervorgeht, fällt der Asche-Tibialängenwert von der vierten zur fünften Versuchswoche, dem Zeitpunkt des Stadiums der Kompensation, mit jetzt einsetzendem verstärktem Wachstum des Knochens, wie die absoluten Werte der Tibialänge ausweisen, in der DEK-Gruppe stark ab.

Es ist bemerkenswert, daß diese Ratten zu einer Kompensation ohne Mithilfe der EK kommen. Die Befunde am Knochen, die das unterschiedliche Verhalten der Ratten mit und ohne Bolusfütterung charakterisieren, müssen wir demnach vornehmlich als unabhängig von den EK ansehen.

Wie verhalten sich nun *die übrigen Drüsen mit innerer Sekretion*? Dieser Frage gingen wir nach, um dem Angriffspunkt des Vitamins D_3 näherzukommen. Bei der Sektion der mit Vitamin D_3 vergifteten Tiere war uns eine Verkleinerung der Hoden, eine hochgradige Atrophie des Thymus und eine Vergrößerung der Nebennieren aufgefallen. Zusammen mit dem Wachstumsstillstand der Ratten dachten wir auch an eine hypophysäre Störung. Die histologische Untersuchung der Hypophyse bestätigte diese Vermutung nicht. Auch die histologische Betrachtung der Hoden ergab zwar eine Hemmung der Ausreifung, die Bildung der Keimzellen war aber bis zu den Spermatocyten erhalten und lebhaft.

Demgegenüber erscheint die *Beziehung der Vitamin D_3-Hypervitaminose zu den Nebennieren* interessant, da sie in unserem Versuch auch ein gewisses Licht auf das Verhältnis dieser Drüsen zu den EK wirft. In der Literatur ist bekannt, daß Nebennierenektomie eine erhöhte Wirkung des EK-Hormons[31] und eine Erhöhung des Blut-Ca hervorruft[32, 37]. Chronische Nebenniereninsuffizienz vermag eine Vergrößerung der EK zu bewirken[18]. EK-xestirpierte Ratten zeigen in der Nebennierenrinde eine vermehrte Lipoidentspeicherung, was auf eine erhöhte Hormonausschüttung der Nebenniere hinweist[11]. In Untersuchungen über das Verhalten der Nierenphosphatase an parathyreopriven und adrenalektomierten Ratten konnten wir auf den gleichartigen Schwund des Fermentes in diesem Organ aufmerksam machen und deuteten den Befund als weiteren Beweis für ähnliche vielleicht sich ergänzende Wirkungen beider Drüsen[12].

Wir haben im vorliegenden Versuch die Nebennieren aller Ratten gewogen und in Prozent des Körpergewichts ausgedrückt, die Werte dem Versuchsverlauf entsprechend in Abb. 12 graphisch dargestellt.

Im Vergleich mit den Normaltieren nimmt bei den mit Vitamin D_3 vergifteten Ratten das *Nebennierengewicht* in der Versuchsfolge deutlich zu, wobei die höher vergifteten Tiere diese Gewichtszunahme stärker zeigen als die niedrig dosierten. Die *Vergrößerung* wird man im Sinne der heutigen Lehre (SELYE) als Folge eines chronischen Streß ansehen, dem sich die Nebenniere anpaßt. Wir bezeichneten dieses Verhalten früher im Zusammenhang mit chronischer Abkühlung als *Kompensationsstadium*[42]. Histologisch entspricht ihm eine verstärkte Lipoidablagerung in allen Schichten der Rinde. Wichtig ist die Feststellung, daß die Nebenniere der parathyreopriven Tiere am stärksten an Gewicht zunimmt. Sollten die Nebennieren für den Hormonausfall der EK zumindest vikariierend eintreten und damit den Ausgleich

(Kompensation) rascher und schneller herbeiführen als bei den nicht parathyreopriven Ratten? Diese Frage ist mit dem vorliegenden Befund noch nicht eindeutig beantwortet, wäre aber am ehesten wohl in diesem Sinne zu klären.

Der *Thymus* zeigt histologisch eine völlige Entleerung der Rinde von Lymphocyten, so daß nur das Reticulum im extremen Fall übrigbleibt. Ob sich hinter diesem Befund mehr als die Folge der allgemeinen körperlichen Belastung und Vergiftung verbirgt, ist nicht ohne weiteres zu sagen. Man könnte in diesem Zusammenhang an die von Nitschke[29] vertretene Ansicht denken, der im lymphatischen Gewebe das Vorhandensein eines Calcinosefaktors vermutet. Wir möchten aber uns auf Einzelheiten in dieser Beziehung nicht einlassen.

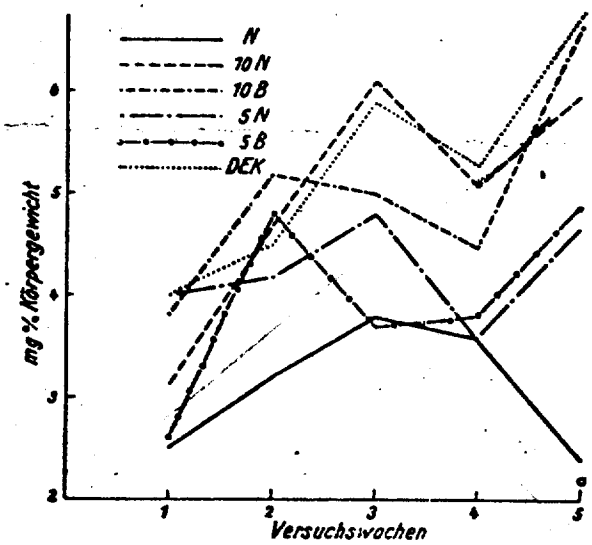

Abb. 12. Nebennierengewicht im Verhältnis zum Körpergewicht

Es bleiben noch die Befunde an den übrigen Organen zu besprechen. Nur die *Veränderungen der Nieren* erscheinen bemerkenswert. Schon makroskopisch fallen sie teilweise durch Vergrößerung und blaßgelbliche Farbe auf, die den Nieren das Aussehen einer Nephrose verleiht. Mikroskopisch findet man in diesen Fällen die in der Literatur bekannte Kalkablagerung bei Vitamin D-Vergiftung. Der Kalk setzt sich unter dem Epithel der gewundenen Harnkanälchen in Form von geschichteten Kugeln ab, die sich vergrößern, das Epithel komprimieren und abdrängen, so daß es abstirbt und die Kalkkonkremente in das Lumen der Kanälchen gelangen. Im abführenden Teil bleiben sie dann z. T. stecken und verlegen die Lichtung, so daß sich die Kanälchen darüber erheblich erweitern und vereinzelt unter Bildung kleiner Narben untergehen. Die Veränderungen erinnern an die früher von uns bei der chronischen Bleivergiftung von Ratten beschriebenen[13]. Wir hielten diese Befunde für eine quantitative Auswertung nicht geeignet.

Wir konnten an den *Schilddrüsen histologisch* keine besonderen Befunde erheben. Eine Beeinflussung der Schilddrüsenfunktion durch Vitamin D ist bekannt[16] und eine Schilddrüsenunterfunktion [29] durch Vitamin D-Gaben aufzuheben. Die Mitwirkung der Schilddrüse bei der Hypervitaminose des Vitamin D ist theoretisch nicht auszuschließen. Man könnte sich den Körpergewichtsverlust nach hohen Vitamin D-Gaben evtl. unter Mitwirkung dieser Drüse vorstellen, zumal wir den unterschiedlichen Körpergewichtsverlust

in den einzelnen Versuchsgruppen nicht allein auf unterschiedlichen Futterverzehr zurückführen konnten und sich in früheren Stoffwechselversuchen[3] kein wesentlicher Einfluß des Bolus alba auf die Verdaulichkeit der Nährstoffe der Diät ergeben hatte.

Nach diesen Ausführungen können wir also annehmen, daß sowohl die *Knochenveränderungen* als auch die zum Test herangezogenen *Veränderungen der EK und Nebennieren* eine *unmittelbare Folge des Vitamin D_3* sind. Somit beruhen die Unterschiede zwischen den unser Thema betreffenden Versuchsgruppen auf einer *direkten Resorptionsförderung für Vitamin D_3 durch Bolus alba*. Diese Resorptionsförderung könnte man sich rein mechanisch infolge besserer Oberflächenverteilung des Vitamin D_3 durch das extramicellare Adsorbens vorstellen. Dem Resorptionsvorgang geht also eine Adsorption auf leicht zugänglichen Oberflächen voraus, während bei den intramicellaren Adsorbentien innere Oberflächenadsorption die Resorption des Adsorbendum durch lokale Unzugänglichkeit behindert. Eine Beeinflussung der Vitamin D_3-Hypervitaminose durch adsorptive Bindung und Resorptionsminderung von Vitamin A durch Bolus alba scheint ausgeschlossen, wie frühere Versuche mit Vitamin A und Bolus bereits zeigten. Die Vitamin A-Aktivität der Diät war zu gering, um die Vitamin D_3-Hypervitaminose überhaupt wesentlich beeinflussen zu können[17, 26, 38]. Die zwischenzeitlich verabreichte Vitamin A-Gabe war dem Einfluß des Adsorbens entzogen. Sie reichte zwar aus, wie wir vermuten und soweit das nicht auch auf die zweitägige Unterbrechung der Vitamin D_3-Applikation zurückzuführen ist, Erholungserscheinungen bei den EK und den Nebennieren auszulösen, doch reichte sie nicht aus, die bestehenden Unterschiede zwischen mit und ohne Bolus gefütterten Ratten zu verdecken.

Andere alimentäre Einflüsse auf die Vitamin D_3-Hypervitaminose, die durch Bolus alba hervorgerufen werden, sind theoretisch denkbar, können aber im Rahmen unseres Experimentes nicht diskutiert werden.

Zusammenfassung

Es wurde bei wachsenden Ratten der alimentäre Einfluß einer Bolus alba-Fütterung auf das Erscheinungsbild der Vitamin D_3-Hypervitaminose untersucht. Als Untersuchungsobjekte dienten Femur, Tibia, Epithelkörperchen, Schilddrüse, Hypophyse, Hoden, Thymus und Nieren. Die Untersuchungen erfolgten mittels chemisch-analytischer, histologischer und röntgenologischer Methoden. Folgende Ergebnisse wurden erzielt:

1. Der Knochen zeigt im Laufe einer fünfwöchigen Versuchsdauer eine wechselnde Veränderung, die über das Stadium der Osteosklerose mit zunehmender Verkalkung zu einem resorptiven Abbau des überschüssig gebildeten Knochens führt. Die letzteren Veränderungen werden als Ausdruck eines Kompensationsvorganges des Organismus gegen die Vitamin D_3-Vergiftung angesehen. Gemessen am Grad der Verkalkung, der Relation Asche % zu Tibialänge in cm und des prozentualen Asche-

gehaltes der fettfreien Trockensubstanz des Knochens sowie dem Grad der Osteosklerose und der Kompensationsvorgänge ergibt sich eine alimentäre Beeinflussung der Vitamin D_3-Hypervitaminose durch Bolus alba, die im Sinne einer Resorptionsverbesserung für das Vitamin D_3 aufzufassen ist.

2. Eine funktionell statistische Auswertung des Gesamtvolumens, der Kerngröße und der Kern-Plasmarelation der Epithelkörperchen bestätigt obiges Ergebnis. Im einzelnen nimmt der Funktionszustand der Epithelkörperchen normaler Ratten mit fortschreitendem Wachstum ab. Diese Funktionsabnahme folgt bei Vitamin D_3-Vergiftung dem Grade der Vergiftungsintensität. Durch Vitamin A-Gaben kann die Funktionshemmung der Epithelkörperchen aufgehoben werden.

3. Die Untersuchung der Hypophyse, der Schilddrüse und Hoden ergibt bei der Vitamin D_3-Vergiftung keinen wesentlichen Befund. Von den übrigen Organen sind nur die Nieren durch Kalkablagerung und Retentionsvorgänge stärker verändert. Mit zunehmender Vergiftung macht sich eine weitgehende Atrophie des Thymus bemerkbar. Die Nebennieren nehmen bezogen auf das Körpergewicht dem Grade der Vergiftung entsprechend an Größe zu. Parathyreoprive Ratten zeichnen sich durch besondere Gewichtszunahme der Nebennieren aus. Bei diesen Tieren erfolgt die Kompensation am Knochen ohne Mithilfe der EK besonders intensiv, wie das histologische Bild und die Relation % Asche zu Tibialänge ausweist.

Somit findet sich zwischen den Versuchsgruppen mit und ohne Bolus alba-Fütterung bei der Vitamin D_3-Hypervitaminose ein alimentär bedingter Unterschied, der auf eine Förderung der Vitamin D_3-Resorption nach oraler Bolusapplikation zurückgeführt wird, wie sich aus dem Verhalten des Knochens und der innersekretorischen Drüsen deutlich ableiten läßt.

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KAOLIN GRANULOMA OF THE STOMACH

ALLAN L. COHN, M.D.
ALFRED S. WHITE, M.D
AND
HELEN B. WEYRAUCH, M.D.
SAN FRANCISCO

The harmful effects on the human body of silica and certain silicates have been known for many years. While references to the lesions in the lung are numerous and the changes caused by silicates in the mucosa, peritoneum and serosa have been described, there have to our knowledge never been recorded the pathologic results of the silica group in the human stomach.

We present herewith a case of granuloma of the stomach which followed ingestion of medication containing colloidal kaolin (hydrated aluminum silicate, $Al_2Si_3O_8 + 5H_2O$) together with the results of preliminary experiments in rabbit stomachs using the same medicinal preparation.

PATHOLOGIC CONDITIONS CAUSED BY SILICA AND SILICATES

Silica and certain silicates cause characteristic reactions in tissue. This is a proliferative type of reaction in which there are nodules with mononuclear or giant cells enveloped by a fibrous tissue beyond which are scattered lymphocytes. There is great similarity of the reaction to that produced by the tubercle bacillus. Numerous authors have described this in detail.[1]

Gardner,[2] demonstrating the similarity of lesions produced by silica and the tubercle bacillus, showed that

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From the Mount Zion Hospital.

Read before the Section on Gastro-Enterology and Proctology at the Ninety-Second Annual Session of the American Medical Association, Cleveland, June 6, 1941.

Dr. Franklin I. Harris, chief of surgery, gave permission to study this case. The pathologic investigations were made under the direction of Dr. G. Y. Rusk, chief pathologist to the Mount Zion Hospital. Mr. John Finn, consulting chemist for the State of California Compensation Insurance Fund and the Newmont Mining Company, collaborated in the mineralogical studies.

1. In addition to the authors referred to in footnotes 2 to 6, descriptions have been published by:
   Miller, J. W., and Sayers, R. R.: The Physiological Response of Peritoneal Tissue to Certain Industrial and Pure Mineral Dusts, Pub. Health Rep. **51**: 1677-1688 (Dec. 4) 1936.
   Kettle, E. H.: Experimental Pneumonoconiosis: Infective Silicatosis, J. Path. & Bact. **38**: 201 (March) 1934.
   Haynes, F.: Experimental Dust Inhalation in Guinea Pigs, J. Hyg. **31**: 96 (Jan.) 1931.
   Carleton, H. M.: The Pulmonary Lesions Produced by the Inhalation of Dust in Guinea Pigs, J. Hyg. **22**: 438 (July) 1924.
2. Gardner, L. U.: The Similarity of the Lesions Produced by Silica and by the Tubercle Bacillus, Am. J. Path. **13**: 13-14 (Jan.) 1937.

the giant cells which develop in the tissues reacting to silica are not of the ordinary foreign body giant cell type. They resemble the Langhans cell of tuberculosis.

Silica (Si_2O), probably most active in colloidal form,[3] produces the typical lesion of silicosis, but certain of the silicates are also active dusts and produce a similar type of reaction. Notable among these are asbestos and talcum powder, magnesium silicate.[4]

Fig. 1.—Gray nodule covered with numerous shiny excrescences revealed by gastroscopy on the posterior wall of the antrum.

Kettle[5] has produced lesions using kaolin. By analysis his specimens of kaolin were shown to contain particles of quartz and numerous fibers of sericite (potassium aluminum silicate), the majority measuring 3 microns or less. Jones, Carleton and Haynes also came to the conclusion that kaolin is a potentially dangerous dust. Whether it is the quartz or the sericite in kaolin which is responsible for the lesions has not been unequivocally determined.[6]

However, Kettle[5] points out, "with different forms of crystalline silica and with certain silicates essentially the same kind of lesion is produced, such modifications as occur depending on the size of the silica particles and their degree of solubility." Gardner[2] also agrees that the size of the particle is very important in the type and extensiveness of the lesion produced.

With this knowledge, namely that silica and certain silicates may produce a specific type of granulomatous reaction in various tissues of the body, we may now consider their effect on the gastric mucosa.

REPORT OF CASE

History.—A white man aged 37, an automobile mechanic, had abdominal pain, pyrosis, nausea and vomiting over a period of eight years. He had no permanent relief from various medications or diet. A diagnosis of duodenal ulcer had been previously made, but roentgenographic studies made subsequently were essentially negative. A later diagnosis of colitis had been made erroneously.

Physical examination revealed nothing of significance excepting epigastric tenderness.

The urine, stool, blood count, sedimentation rate and Wassermann reaction were normal. The gastric analysis revealed free hydrochloric acid 68-86 and total acid 77-102.

A gastroscopic examination was made with the 50 degree angle flexible gastroscope. The mucosa of the prepyloric region was thickened. A gray nodular area approximately 2 cm. in diameter was present on the posterior wall of the antrum immediately distal to the angulus (incisura). This nodule was rounded and irregular; the surface was covered with numerous shiny excrescences (fig. 1).

The rugae of the midportion of the body of the stomach were hypertrophied.

Roentgen examination two days later revealed a mild degree of hypertrophic gastritis and duodenitis. The lung fields were clear.

Despite the essentially negative findings, it was thought that the long history of symptoms and the unusual gastroscopic observations were indications for surgery. A subtotal gastrectomy was done five days later.

Gross examination of the stomach after its removal showed evidence of an old healed duodenal ulcer. No lesion as described gastroscopically was seen, much to our chagrin.

The patient became asymptomatic and has remained so for over two and one-half years.

During the early postoperative period we awaited with anxiety the histologic report.

Microscopic Study.—At the base of the mucosa were a few irregularly spaced lesions of a granulomatous nature. These were made up of nodules of a definite achitecture. The center of the nodule contained a large cell which was multinucleated, granular and usually fairly large. The nuclei of many of the giant cells tended to congregate about the periphery.

Encapsulating this cell was a delicate fibrous tissue which was not usually excessive in amount. Beyond the connective tissue might be seen plasma cells, lymphocytes and eosinophilic leukocytes. They were not present in sufficient numbers to constitute a real zone.

In a few of the giant cells there was foreign material (figs. 2 and 3). This varied in size and shape and was colorless and refractile. Some of the particles looked like laminated spicules. Occasionally, fragmented material took a blue stain with hemotoxylin.

Fig. 2.—Nodular areas near the base of the mucosa. In the central part of the nodules giant cells are present, some of which surround foreign material.

Fat, Masson, Kossa and acid-fast stains were negative.

Mineralogical Analysis.—A study was made of the crystals in the giant cells and in the residue of tissue after microincineration. They were insoluble in water, hydrochloric acid

3. Koppenhofer, G. F.: Studies on the Pathogenesis of Silicotic Changes: IV. Tissue Changes After Experimental Introduction of Colloidal Silicic Acid, Virchows Arch. f. path. Anat. **297**: 271, 1936. Emmons, R. C., and Fries, C.: Aluminum and Silicosis, Am. Mineralogist **23**: 654-660 (Oct.) 1938.

4. Antopol, William: Lycopodium Granuloma: Its Clinical and Pathological Significance Together with a Note on Granuloma Produced by Talc, Arch. Path. **16**: 326-330 (Sept.) 1933. Feinberg, Robert: Two Cases of Granuloma Caused by Crystals of Talc, ibid. **24**: 36 (July) 1937. Ramsey, T. L., and Douglass, F. M.: Granulomatous Inflammation Produced by Foreign Body Irritants, J. Internat. Coll. Surg. **3**: 3-10 (Feb.) 1940. McCormick, E. J., and Ramsey, T. L.: Postoperative Peritoneal Granulomatous Inflammation Caused by Magnesium Silicate, J. A. M. A. **116**: 817 (March 1) 1941.

5. Kettle, E. H.: The Interstitial Reactions Caused by Various Dusts and Their Influence on Tuberculous Infections, J. Path. & Bact. **35**: 395 (May) 1932.

6. Dawson, L.: The Incidence of Quartz and Sericite Particles in Silicotic Residues of Silicotic Lungs, J. Hyg. **39**: 405-412 (July) 1939. Jones, W. R.: Silicotic Lungs: The Minerals They Contain, ibid. **33**: 307 (April) 1933. Kettle

and nitric acid. The crystals varied in size from 2 to 4 microns and belong to the hexagonal system. They were anisotropic or doubly refractive. The Becker line showed them to have a higher refractive index than Canada balsam (1.54). They had the same general morphologic appearance as the crystals present in the colloidal kaolin examined and the crystals present in the preparation used by the patient. They had the characteristics of silica.

Fig. 3.—Type of giant cell typical in silicosis surrounding foreign material. The nuclei tend to congregate around the periphery. The material is fragmented, laminated, colorless and refractile. Some of it stains blue with hematoxylin.

Preliminary Experiments on Rabbits.—With the medicament which the patient had been taking, a test experiment on rabbit stomach was undertaken. The preparation consisted of English kaolin colloidal 20 per cent and aluminum hydroxide 2.5 per cent in liquid petrolatum. We report here the findings of this "scout" experiment.

Two series of animals were tested. In the first series 2 cc. of the medication was injected into the gastric mucosa of 3 rabbits.

In series 2 a small area of the stomach mucosa of 4 rabbits was lightly curetted and the animals were fed daily a diet into which was incorporated powdered colloidal kaolin.

The rabbits of series 1 were killed at one, one and one-half and four months respectively after injection. Gross mucosal elevations or nodules were present. The microscopic examination showed groups of giant cells at the periphery of the injected material.

All the giant cells had multiple nuclei, many of which tended to group themselves peripherally or at the end of the cell like those of a Langhans cell. Colorless refractile crystals were present in many of the giant cells (fig. 4).

In the scarified series the same type of reaction was found; in fact there was greater similarity to the human case in these animals. No gross mucosal lesions were found in these animals. However, there were microscopic changes in the submucosa. Multinucleated giant cells occurred singly or in groups and were encapsulated by a fine loose connective tissue, surrounded by lymphocytes, plasma cells and eosinophilic neutrophils. Some of the giant cells contained foreign material. The most striking particles were sharp pointed clear refractile spicules.

There were giant cells about the suture material, but they were of the true foreign body type, which has little resemblance to the specific kind observed in silicosis.

The foreign material had the colorless glassy refractile appearance which characterized some of the particles of the kaolin which was examined in water suspension under the microscope for comparison.

COMMENT

Evidence has been presented to permit the reasonable conclusion that kaolin may cause a granulomatous inflammatory reaction in the stomach. This reaction is similar to that which is typical of the silica group.

Many unknown factors prevent any additional conclusions. We should like to know the conditions necessary for the silicates to produce this inflammatory reaction in the stomach. We should like to know how common this disease is. If the known reaction to silica and to silicates was accidentally discovered in this 1 case, might it not be reasonable to presume that there may be other undiagnosed cases?

We might perhaps be warranted in suggesting that patients using similar preparations be carefully observed and with the gastroscope. If symptoms should occur suggesting gastrointestinal irritation or if no relief should be obtained from the original symptoms, the possible deleterious effects of this group should be considered.

This case also illustrates the value of gastroscopy followed by tissue studies.

SUMMARY

A histopathologic and mineralogical investigation was made of the stomach of a patient who had been ingesting a preparation containing kaolin for a long period of time. Clinical and roentgen examinations revealed no gastric disease. Gastroscopy showed a prepyloric tumor, which however was not seen in the gross resected stomach. Microscopic studies proved the lesion to be a granuloma, with the type of foreign body giant cell present in the lesions of silicosis. Mineralogical studies confirmed the presence of silica in the gastric tissues.

In experimental studies on rabbits it was possible to reproduce the lesions typical of silica irritation which were similar to those found in the human case.

Fig. 4.—Type of giant cell in the rabbit stomach into which the kaolin containing medication of the patient was injected is large with numerous nuclei at the periphery of the cells. Several colorless refractile crystals are seen.

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EFFECT OF CYSTEINAMINE ON EXPERIMENTAL ARTHRITIS
(Action De La Cysteinamine Sur Une Arthrite Experimentale)

By

R. Coulon, R. Charlier, and L. Vandersmissen

of

Research Laboratories, Department of Pharmacodynamics, Societe des Laboratoires Labaz, Brussells

from

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Translation from French

EFFECT OF CYSTEINAMINE ON EXPERIMENTAL ARTHRITIS

R. Coulon, R. Charlier, and L. Vandersmissen

We know, from studies by Bacq and his school, that cysteinamine has many interesting pharmacodynamic properties, aside from its protective effect against ionizing radiation; among these properties, Lecomte and Bohrenstayn [1] have determined an anti-inflammatory action. These authors studied the action of cysteinamine and its oxidized derivative on the tuberculin reaction and on Ambrose and Eds test.

In the first case, they found that the intradermal injection of 1 mg of cystinamine or 3 mg of cysteinamine will weaken the human reaction to tuberculin injected 2 min after the product under study; this weakening was observed in 75% of the cases.

Regarding the Ambrose and Eds test, the authors reported that the rapid intravenous injection of 75 mg/kg cysteinamine will significantly delay the appearance of trypan blue in the dermis. This delay amounts to 190-480% of the value in controls. This anti-inflammatory effect will disappear after 30-40 min. The effect is assumed to be due to the presence of the substance itself in the circulating blood or of the reducing agents which it liberates.

Lecomte et al. [2] also observed that cysteinamine and its oxidized derivative will reduce the edema induced in the rat by an ovalbumin injection, and they found this substance more effective than cortisone in this respect.

The authors concluded from their experiments that cysteinamine and cystinamine possess anti-inflammatory properties both in a delayed reaction of the tuberculin allergy type and in an immediate non-specific lesion due to a chemical irritant.

Based on these observations, we attempted to determine whether

cysteinamine may affect the evolution of inflammatory phenomena caused by the intra-articular injection of an irritant in the rat.

Experimental Technique

Experimental arthritis may be provoked by various methods; Selye [3] suggested a periarticular injection of formalin, and Coutu [4] a suspension of powered mustard.

However, we preferred Ducommun's technique [5] which consists of injecting a sterile 10% suspension of kaolin under the aponeurosis of the tarsotibial joint. This method is assumed to have the advantage of producing a uniform inflammatory reaction which is more granulomatous and less necrotic than the former methods.

We did introduce a slight modification into the technique by injecting directly into the joint rather than into the periarticular tissues. The inflammatory reaction thereby appears an hour after the injection and consists in considerable swelling of the joint region accompanied by changes in skin color. As a criterion we selected the average of the anteroposterior and laterolateral diameters of the right tarsotibial joint, measured before the kaolin injection, one hour after the injection, and every 24 h thereafter.

For our experiment we used 4 groups of animals which were treated as follows:

1) 5 control rats which received an intra-articular injection of 0.2 ml of the 10% kaolin suspension,
2) 5 rats, which received, 1 h after the kaolin injection, an intraperitoneal cysteinamine* dose of 50 mg/kg; the same cysteinamine dose was then repeatedly given every 24 h,

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* We used the cysteinamine sold as Bécaptan by the Société des Laboratoires Labaz, Brussels.

Table. Effect of cysteinamine on experimental arthritis; changes in the diameter of the tarsotibial joint; zero day: reference diameters before the intra-articular kaolin injection; zero day + 1 h after the kaolin injection

Key: 1)... group; 2) days; 3) 0 + 1 h

Days	1st GROUP					2nd GROUP				3rd GROUP				4th GROUP				
	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 2	Rat 3	Rat 4	Rat 5	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5
	6,55	6,45	6,20	5,80	6,30	6,40	6,40	6,40	5,70	—	5,65	6,00	5,85	5,40	5,95	6,30	6,25	6,25	6,80
+ 1 h	7,90	7,35	7,25	7,40	7,25	7,05	7,15	6,95	6,90	—	6,50	7,00	7,25	6,45	6,55	6,65	7,60	6,45	7,10
	9,35	9,75	9,05	9,45	8,80	8,05	8,35	8,25	7,75	—	8,00	8,65	8,30	7,05	8,35	8,75	8,80	8,60	9,50
2	9,70	10,05	9,65	9,75	9,55	8,20	8,25	7,80	8,15	—	7,60	8,25	8,15	6,75	7,80	8,55	8,30	7,95	9,05
3	10,30	11,05	10,70	11,10	10,00	7,80	7,95	8,20	8,10	—	6,70	8,00	7,70	6,80	7,65	8,20	7,95	8,00	8,75
4	9,95	11,15	10,70	11,70	10,00	7,95	8,20	8,00	8,05	—	6,85	8,05	7,90	6,30	7,65	7,70	8,15	7,95	8,55
5	9,95	10,90	10,05	12,15	9,75	7,95	8,15	7,90	7,85	—	6,80	7,80	7,75	6,15	7,30	7,80	7,75	7,55	8,60
6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	7,20	7,30	7,75	7,60	8,55
7	9,40	9,95	9,70	12,30	9,10	7,90	8,00	8,20	7,85	—	6,85	7,00	7,90	6,20	7,15	6,90	7,85	7,50	8,30

3) 5 rats receiving the first cysteinamine dose 24 h after the kaolin injection, then the same cysteinamine dose every 24 h thereafter,

4) 5 rats pretreated with cysteinamine 48 h before the kaolin injection at the rate of one injection every 24 h at the same dose, and then, as for group 3, with an identical injection every 24 h.

Results

(see Figure 1 and Table)

First Group (controls): 1 h after the kaolin injection, the average joint diameter increased from 6.26 mm to 7.43 mm; after 24 h, it measured 9.28 mm, reaching a maximum, i.e. 10.7 mm, on the 4th day.

The skin is purple, the joint taut, and the periarticular tissues exhibit considerable edema.

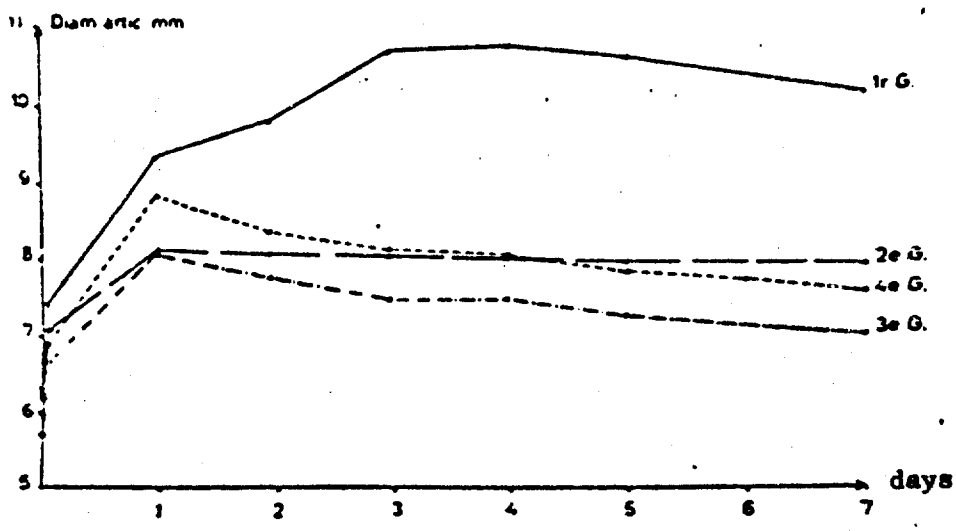

FIG. 1

2nd Group: 1 h after the kaolin injection, the average diameter increased from 6.19 mm to 7.07 mm; 24 h after the cysteinamine administration, this

diameter measure only 8.11 mm. From then on, swelling of the joint decreased in all the animals of this group, and on the 4th day, the average diameter was only 7.97 mm. The macroscopic aspect also differed from that in the controls: the skin had retained its normal color, and there was markedly less edema.

3rd Group: 1 h after the kaolin injection, the average diameter increased from 5.68 mm to 6.66 mm, and 24 h later to 8.06 mm; the macroscopic aspect resembled that of the controls. When measured 24 h after the cysteinamine injection, the diameter had dropped to 7.76 mm, and the macroscopic signs were considerably improved. On the 4th day, the diameter was only 7.23 mm.

4th Group: 1 h after the kaolin administration, the diameter increased from 6.31 mm to 6.87 mm, and 24 h later to 8.8 mm. Starting with the 2nd day, the reduction of volume proceeded as for the animals of the 2nd and 3rd group.

Conclusions

1) The intraperitoneal injection of cysteinamine at a 50 mg/kg dose, given under the above experimental conditions, considerably reduced the inflammatory reaction induced in the rat by the intra-articular injection of a sterile kaolin suspension. While the average diameter of the joint decreased gradually in the treated rats 24 h after the kaolin injection, this diameter increased in the control rats up to the 4th day.
2) The preventive treatment failed to inhibit the development of the inflammatory signs, nor did it improve the favorable effect of the post-injection treatment.

According to Bacq, Fischer and Pirotte [6], cysteinamine did not cause any drop in the eosinophil count, did not increase the elimination of reducing corticoids or any change in the uric acid/creatinine ratio. Hence the activity of this molecule in experimental arthritis cannot be attributed to an ACTH-like action.

The absence of any preventive cysteinamine effect could find its explanation in the rapidity with which this product is metabolized and excreted [7]. We are conducting additional experiments to determine whether more frequent cysteinamine injections (every 2 h after the kaolin injection) might increase the favorable effect.

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Arch. int. pharmacodyn., 1954, XCIX, 3-4.

SOCIÉTÉ DES LABORATOIRES LABAZ (BRUXELLES) (1)
LABORATOIRES DE RECHERCHES — DÉPARTEMENT DE PHARMACODYNAMIE

ACTION DE LA CYSTÉINAMINE SUR UNE ARTHRITE EXPÉRIMENTALE

PAR

R. COULON, R. CHARLIER ET L. VANDERSMISSEN

(Travail reçu le 1-6-1953).

Nous savons par les travaux de BACQ et de son école que, outre son activité protectrice vis-à-vis des radiations ionisantes, la cystéinamine possède de nombreuses propriétés pharmacodynamiques intéressantes, parmi lesquelles LECOMTE et BOHRENSTAYN (1) ont mis en évidence une action anti-inflammatoire. Ces auteurs ont observé l'action de la cystéinamine et de son dérivé oxydé sur la réaction à la tuberculine et le test D'AMBROSE et de EDS.

Dans le premier cas, ils ont constaté qu'après injection dans le derme de 1 mg de cystinamine ou de 3 mg de cystéinamine, la réaction à la tuberculine, injectée dans le derme deux minutes après le produit étudié, était affaiblie chez l'homme, dans 75 % des cas.

En ce qui concerne le test d'AMBROSE et de EDS, les auteurs constatent que l'injection intraveineuse rapide de 75 mg/kg de cystéinamine provoque un retard net à l'apparition du bleu trypan dans le derme. Ce retard varie de 190 à 480 % de la valeur témoin. Cette action anti-inflammatoire devient nulle après 30 à 40 minutes. Cette action serait liée à la présence dans le sang circulant de la substance elle-même ou des réducteurs qu'elle libère.

LECOMTE et coll. (2) ont également observé que la cystéinamine et son dérivé oxydé diminuent l'oedème provoqué chez le rat par l'injection d'ovalbumine et que, à cet égard, la cystéinamine est plus efficace que la cortisone.

(1) Address : Avenue de Bajus 3, ~~Woluwe-Saint-Lambert, Bruxelles~~

De leurs expériences les auteurs concluent que la cystéinamine et la cystinamine sont douées de propriétés anti-inflammatoires aussi bien sur une réaction différée du type allergie tuberculinique que sur une lésion immédiate irritative chimique non spécifique.

Nous inspirant de ces observations, nous avons recherché si la cystéinamine pouvait influencer l'évolution des phénomènes inflammatoires dûs à l'injection intraarticulaire d'une substance irritante chez le rat.

Technique

Il existe différentes méthodes pour provoquer une arthrite expérimentale; Selye (3) a préconisé l'injection périarticulaire de formaline et Coutu (4) d'une suspension de poudre de moutarde.

Nous avons cependant préféré la technique de Ducommun (5) qui consiste à injecter sous l'aponévrose de l'articulation tibiotarsienne une suspension stérile de kaolin à 10 %. Cette méthode présenterait l'avantage de produire une réaction inflammatoire uniforme, plus granulomateuse et moins nécrosante que les précédentes.

Nous avons cependant légèrement modifié la technique d'injection en pratiquant celle-ci directement dans l'articulation plutôt que dans les tissus périarticulaires. Les phénomènes inflammatoires se manifestent dès l'heure qui suit l'injection par un gonflement important de la région articulaire accompagné de modifications de teinte de la peau. Comme critère, nous avons pris la moyenne des diamètres antéropostérieur et latérolatéral de l'articulation tibiotarsienne droite. Les mesures sont faites d'abord avant l'injection de kaolin puis 1 heure après et, par la suite, toutes les 24 heures.

L'expérience a porté sur 4 groupes d'animaux se répartissant comme suit :

1) 5 rats témoins recevant une injection intra-articulaire de 0,2 ml de la suspension à 10 % de kaolin.

2) 5 rats recevant 1 heure après l'injection de kaolin, une dose de 50 mg de cystéinamine [1] par kg de rat, par voie intrapéritonéale. La même dose de cystéinamine est répétée toutes les 24 heures.

3) 5 rats recevant la première dose de cystéinamine 24 heures après l'injection de kaolin, dose qui est répétée toutes les 24 heures.

[1] La cystéinamine utilisée est le Bécaptan de la Société des Laboratoires Labaz, Bruxelles.

Influence de la cystéinamine
par ka

Modifications du diamètre mo

Au jour o : diamètres de référence

Au jour o + 1 H

Jours	1er groupe					2me groupe				
	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5
o	6,55	6,45	6,20	5,80	6,30	6,40	6,40	6,40	5,70	6,05
o + 1 H.	7,90	7,35	7,25	7,40	7,25	7,05	7,15	6,95	6,90	7,30
1	9,35	9,75	9,05	9,45	8,80	8,05	8,35	8,25	7,75	8,15
2	9,70	10,05	9,65	9,75	9,55	8,20	8,25	7,80	8,15	7,95
3	10,30	11,05	10,70	11,10	10,00	7,80	7,95	8,20	8,10	8,00
4	9,95	11,15	10,70	11,70	10,00	7,95	8,20	8,00	8,05	7,65
5	[illegible]	10,90	10,05	12,15	9,75	7,95	8,15	7,90	7,85	7,75
6	—	—	—	—	—	—	—	—	—	—
7	9,40	[illegible]	[illegible]	[illegible]	[illegible]	7,90	[illegible]	[illegible]	7,85	[illegible]

arthrite expérimentale
le rat

l'articulation tibio-tarsienne
m)

ction intraarticulaire de Kaolin

H après l'injection de kaolin

3me GROUPE					4me GROUPE				
at 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5
5,50	5,65	6,00	5,85	5,40	5,95	6,30	6,25	6,25	6,80
6,10	6,50	7,00	7,25	6,45	6,55	6,65	7,60	6,45	7,10
8,30	8,00	8,65	8,30	7,05	8,35	8,75	8,80	8,60	9,50
1,05	7,60	8,25	8,15	6,75	7,80	8,55	8,30	7,95	9,05
1,10	6,70	8,00	7,70	6,80	7,65	8,20	7,95	8,00	8,75
1,20	6,85	8,05	7,90	6,30	7,65	7,70	8,15	7,95	8,55
1,45	6,80	7,80	7,75	6,15	7,30	7,80	7,75	7,55	8,60
[illegible]	—	—	—	—	7,20	7,30	7,75	7,60	8,55
[illegible]	6,85	7,00	7,90	6,20	7,15	6,90	7,85	7,50	8,30

4) 5 rats ayant reçu pendant les 48 heures précédant la création de l'arthrite une injection par 24 heures de la même dose de cystéinamine puis, comme pour le 3e lot, une injection identique toutes les 24 heures.

Résultats

(Voir figure 1 et tableau)

1er *Groupe (Rats témoins):* Une heure après l'injection de kaolin, le diamètre articulaire moyen est passé de 6,26 mm à 7,43 mm. 24 heures après il est de 9,28 mm, le maximum, 10,7 mm, étant atteint au 4me jour.

La peau présente une coloration nettement violacée, l'articulation est tendue et les tissus périarticulaires sont fortement oedématiés.

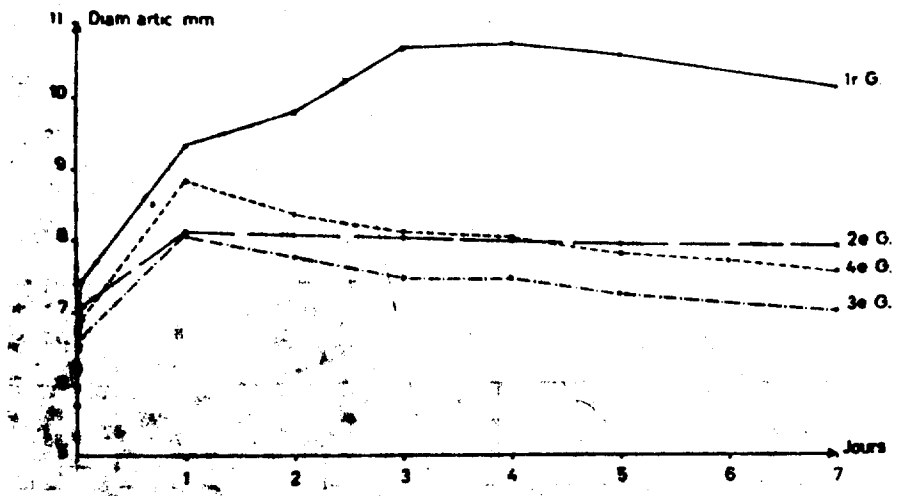

Fig. 1

2me *Groupe:* Une heure après l'injection de kaolin, le diamètre moyen est passé de 6,19 mm à 7,07 mm. 24 heures après l'administration de cystéinamine, le diamètre est seulement de 8,11 mm. A partir de ce moment tous les animaux de ce groupe accusent une nette diminution du gonflement de l'articulation dont le diamètre moyen n'est plus au 4me jour que de 7,97 mm. L'aspect macroscopique est nettement différent de celui des témoins, la peau a gardé une coloration normale

3me *Groupe:* Une heure après l'injection de kaolin, le diamètre moyen passe de 5,68 mm à 6,66 mm et 24 heures après à 8,06 mm, l'aspect macroscopique est semblable à celui des témoins. 24 heures après l'injection de cystéinamine, le diamètre tombe à 7,76 mm et les signes macroscopiques s'améliorent considérablement.

Au 4me jour, le diamètre n'est plus que de 7,23 mm.

4me *Groupe:* Une heure après l'administration du kaolin, le diamètre passe de 6,31 mm à 6,87 mm, puis 24 heures plus tard à 8,8 mm. A partir du 2me jour, la diminution de volume évolue de la même manière que chez les animaux des 2me et 3me groupes.

Conclusions

1) L'injection intrapéritonéale de cystéinamine à la dose de 50 mgr/kg, faite dans les conditions expérimentales précitées, réduit dans de notables proportions les réactions inflammatoires provoquées chez le rat par l'injection intraarticulaire d'une suspension stérile de kaolin. En effet, tandis que le diamètre articulaire moyen diminue graduellement chez les rats traités à partir des 24 heures qui suivent l'injection de kaolin, les rats témoins voient ce diamètre augmenter progressivement jusqu'au 4me jour.

2) Le traitement préventif n'a pu empêcher le développement des phénomènes inflammatoires et n'a pas amélioré l'effet favorable du traitement institué après l'injection de kaolin.

Selon les travaux de Bacq, Fischer et Pirotte (6), la cystéinamine ne provoque ni chute des éosinophiles, ni augmentation de l'élimination des corticoïdes réducteurs, ni modification du rapport $\frac{\text{acide urique}}{\text{créatinine}}$.

On ne peut donc attribuer à une action ACTH-like l'activité de cette molécule vis-à-vis de l'arthrite expérimentale.

L'absence d'action préventive de la cystéinamine pourrait s'expliquer par la rapidité du métabolisme et de l'excrétion du produit (7). Des essais complémentaires sont en cours pour déterminer si des injections de cystéinamine plus fréquentes (notamment toutes les 2 heures après l'injection du kaolin) peuvent avoir un effet encore plus favorable.

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The Value of Bentonite for Diarrhea

FREDERIC DAMRAU, M.D.
New York City

IN A GROUP of 35 cases of acute diarrhea of diverse etiology, the use of a special preparation of bentonite* provided substantial relief in 34 cases (97 per cent). The causative factors included virus infections, food allergy, spastic colitis, mucous colitis, and food poisoning.

NATURE OF BENTONITE

Bentonite is a native, colloidal, hydrated aluminum silicate. It occurs as a very fine, odorless, pale buff or cream-colored powder, free from grit, and has a slightly earthy taste. Although it is insoluble in water, it swells to approximately 12 times its volume when added to water. Bentonite magma is prepared by adding 50 Gm. of bentonite to a sufficient quantity of purified water to make 1 liter.[1]

Geologically, bentonite is a rock composed of clay minerals formed by the alteration of minute glass particles that once composed volcanic ash. The name was derived from the Fort Benton series of cretaceous rocks in Wyoming, where it was first found.[2]

Bentonite has been used extensively, in the form of a gel, as a bulk laxative and also as a base in many dermatologic formulations.[3] A desirable property of bentonite is its avid adsorption of other substances, both organic and inorganic.[4]

HISTORY OF MEDICAL USES

Hydrated aluminum silicate was used for centuries in China for summer diarrheas and cholera.[5] In 1712 Father Deutrecolle, a Jesuit missionary, described the clay works in China and mentioned that the clay was used in treating diarrhea.[6]

The use of hydrated aluminum silicate with other medications during the Balkan war of 1910 reduced the mortality from cholera among the soldiers from 60 to 3 per cent, and it also proved valuable in the 1919 epidemic of cholera in China.[7] In India hydrated aluminum silicate was found useful in the treatment of acute bacterial food poisonings encountered in the British Army.[8]

Hydrated aluminum silicate has been used as an adsorptive in the symptomatic treatment of various forms of enteritis[9] including ulcerative colitis.[10]

Gastrointestinal adsorbents including hydrated aluminum silicate are presently recommended in acute diarrhea and bacillary dysentery to adsorb the toxins which produce the diarrhea.[11] Hydrated aluminum silicate has been used in the treatment of abnormal intestinal fermentation to adsorb gases, toxins and bacteria.[12] In a fluid medium it carries down large numbers of bacteria and adsorbs the toxins of cholera, typhoid, dysentery, and apparently of the putrefactive and proteolytic bacteria.[12]

In vitro studies suggest that the beneficial action of hydrated aluminum silicate in cholera depends on the adsorption of the cholera toxins and inclusion of the bacteria.[6] Later investigations indicate that bentonite adsorbs certain viruses including those of intestinal influenza, as suggested by my successful results in that disease. Bacteriologically bentonite is used for purification of viruses.[14, 15]

* The bentonite preparation (with distilled water as a vehicle) used in this study was Vit-Ra-Tox "16", supplied by V. E. Irons, Inc., of Boston, Mass.

The specific properties of bentonite have been shown to have an important bearing on its therapeutic uses.[16] A paste made from hydrated aluminum silicate is recommended by Russian radiologists to deactivate hands contaminated by radium salts.[17] In Russia bentonite has been used in the therapy of peptic ulcer.[18]

Since bentonite has such strong adsorptive powers its consumption in excessive amounts over a considerable period of time could render vitamin A and other necessary nutrients unavailable by adsorbing them from the alimentary canal. Independent experiments purposely designed to determine how much this adsorption would adversely affect the growth and health of experimental animals indicated no ill effects when the intake of bentonite did not exceed 25 per cent of the total diet.[19]

Present Study

The therapeutic efficacy of bentonite for acute diarrhea of diverse etiology was evaluated in a group of 35 cases. The causative factor was virus infection in 18 cases, food allergy in 8, spastic colitis in 4, mucous colitis in 3, and food poisoning in 2.

The symptoms were diarrhea in 35 cases (watery stools in 29, soft stools in 6), abdominal cramps in 30, anorexia in 18, malaise in 15, headache in 14, nausea in 13, and weakness in 7.

The group included 25 females and 10 males. The average age was 51.8, with a range from 23 to 76 years. The average weight was 153.2 lbs., with a range from 92 to 260 lbs.

A complete physical and routine urinalysis were made in every case to eliminate concomitant diseases which might affect the course of the diarrhea. Where indicated, laboratory and X-ray examinations were performed as part of the diagnostic procedure. Every effort was made to obtain a homogeneous group of patients so as to eliminate variables from the study.

The standard treatment employed was 2 tablespoonfuls of bentonite in distilled water as a vehicle 3 times daily. In cases of food allergy the dosage was increased above 6 tablespoonfuls daily.

Therapeutic Results

Acute diarrhea was relieved by bentonite in 34 of the 35 cases (97 per cent) in an average period of 3.8 days, ranging from 1 to 14 days. Following treatment the number of bowel movements per diem was reduced from an average of 4.0 (range 2 to 6) to an average of 1.8 (range 1 to 3).

In the 18 cases of diarrhea due to virus infection the therapeutic response was unusually prompt. Hence in this group the average duration of treatment was 2.2 days with a range of 1 to 3 days. In the 8 cases due to food allergy the diarrhea persisted longer and on many occasions returned if the same allergenic food was eaten again.

The concomitant symptoms were relieved in the following case percentages: abdominal cramps in 24 of 30 cases (80 per cent), anorexia in 14 of 18 cases (78 per cent), malaise in 12 of 15 cases (80 per cent), headache in 10 of 14 cases (71 per cent), nausea in 11 of 13 cases (85 per cent), and weakness in all of the 7 cases (100 per cent).

No side-effects attributable to the medication were observed in any case. Routine chemical and microscopic examination of the urine was negative in all 35 cases.

Summary

A preparation of bentonite with distilled water as a vehicle was found to be safe and highly effective in the treatment of acute diarrhea. The diarrhea was relieved in an average of 3.8 days in 34 of 35 cases (97 per cent) of diverse etiology. The number of bowel movements per diem was reduced from an average of 4.0 to an average of 1.8. The concomitant symptoms were also effectively relieved in most cases, namely, abdominal cramps (80 per cent), anorexia (78 per cent), malaise (80 per cent), headache (71 per cent), nausea (85 per cent), and weakness (100 per cent).

Fastest results were observed in intestinal influenza, in which the diarrhea was controlled in an average of 2.2 days.

Bentonite is a native, colloidal, hydrated aluminum silicate. A survey of medical literature disclosed that hydrated aluminum silicate was used for centuries in China for summer diarrheas and cholera. Favorable results have been reported in cholera, bacillary dysentery, acute bacterial food poisonings, ulcerative colitis, and various forms of enteritis.

It has been established in vitro and in vivo that hydrated aluminum silicate adsorbs toxins, bacteria and viruses. This property helps to explain its therapeutic usefulness in acute diarrhea of diverse etiology.

By virtue of its physical action bentonite serves as an adsorbent aid in detoxification of the intestinal canal.

2 Tudor City Place
New York 17, N. Y.

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Silicates in Broiler Diets[1]

ELBERT J. DAY, R. D. BUSHONG, JR. AND BEN C. DILWORTH
Department of Poultry Science, Mississippi State University, P. O. Box 5188, State College, Mississippi 39762

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THE silicates, more commonly known as bentonite, kaolins, vermiculite and other clays, have been used in animal feeds and industrially for many years. They are used in feeds principally because of their ability to enhance pellet durability. As a group, they often are referred to as "pellet binders". Eventually, they may be widely used to control "watery droppings" in poultry. The highly adsorptive characteristic of binders led to experiments in this area (Quisenberry and Bradley, 1964). These workers found that sodium bentonite at both the 2.5 and 5.0% dietary levels significantly reduced the moisture content of the droppings of cage layers. Similarly, they have been proposed by some for the prevention of "loose droppings" in broilers where such conditions cause increased condemnation in dressing plants.

The highly adsorptive nature of the silicates led to studies to determine whether or not they adversely affect nutrient availability (Briggs and Spivey, 1954; Laughland and Phillips, 1954). These workers found that bentonite could create a vitamin A deficiency under certain conditions where purified diets were employed. However, recent data obtained with practical diets indicate that silicates enhanced feed efficiency or caloric efficiency (Cage layers; Quisenberry and Bradley, 1964: Turkeys; Almquist *et al.*, 1967: Broilers; Kurnick and Reed, 1960; Ousterhout, 1966). Several of the above research groups found that the silicates may act in slowing feed passage time through the digestive system. This slowing action has been postulated to be the explanation as to how they improve nutrient utilization by longer passage time

[1] Mississippi Agricultural Experiment Station Journal Article No. 1799.

allowing for greater digestion and absorption.

The recent favorable reports about silicates, along with the well known fact that they improve pellet quality, prompted the experiments reported herein. Three experiments were conducted to study the effect of source and dietary level of silicates on broiler performance.

EXPERIMENTAL

Two kaolin[2] binders and one sample of calcium bentonite[3] were tested in this study. One large floor experiment (Exp. 2) and two battery (Exps. 1 and 3) studies were conducted. The test diets in Experiments 1 and 3 were each fed to replicate lots of 25 broiler cockerels. Replicate lots of 80 straight-run broiler chicks were fed each diet in Experiment 2. The floor study was of eight weeks (0–8 wks.) duration, while the birds were fed the experimental diets from 0–4 weeks of age in the battery experiments. Vantress × Cobb male broiler chicks were used in the battery tests and straight-run chicks of the same cross were used in the floor study. Individual body weights and group feed consumption data were obtained at the termination of each experiment. The experimental diets and tap water were provided *ad libitum*.

The binders were added at the expense of the total diet in all instances in order to maintain nutrient balance. The basal diets are shown in Table 1. All diets were fed in the mash form. Dietary levels of the binders are shown in the summary tables along with the results from each experiment. Caloric efficiency per pound of gain was calculated for each treatment in an attempt to more clearly evaluate the results obtained with each diet. Caloric efficiency in this study implies simply the productive feed calories required per kilogram of broiler weight.

The performance data, growth rate and feed utilization were analyzed statistically by the analysis of variance (Snedecor, 1957) with significant differences between treatment means determined using the multiple range test (Duncan, 1955).

TABLE 1. *Composition of basal diets*

Ingredient	Battery study Exps. 1 & 3	Floor study, Exp. 2 Starter 0–5 wk.	Finisher 5–8 wks.
Yellow corn	56.95	58.95	62.95
Soybean meal, 50% protein	30.00	29.87	24.37
Fish meal, peruvian	4.00	4.00	4.00
Alfalfa meal, 17% protein	1.50	1.50	2.00
Poultry oil	5.00	3.00	4.00
Dical. phos., 18.5% P, 26% Ca	1.10	1.20	1.20
Limestone	0.75	0.85	0.85
Salt	0.40	0.30	0.20
DL-methionine, 98%	0.05	.035	.035
Broiler premix[1]	0.25	0.25	0.25
Coccidiostat premix[2]	-	0.05	0.05
Total	100.00	100.00	100.00
Calculated analysis:			
Crude protein, %	22.80	22.99	20.66
Energy, prod. Cal./kilogm.	2357	2284	2357
Energy, met. Cal./kilogm.	3179	3102	3188
Calcium, %	.843	0.90	0.89
Total phos., %	.659	0.67	0.65
Avail. phos., %	.417	0.44	0.43
Lysine, %	1.3082	1.3106	1.1516
Methionine, %	.4812	.4338	.4037
Meth.+cystine, %	.8411	.7982	.7349

[1] Furnished the following per kilogram of diet: Vitamin A, 5,516 I.U.; vitamin D_3, 1,653 I.C.U.; vitamin E, 1.1 I.U.; riboflavin, 4.4 mg.; niacin, 27.6 mg.; d-pantothenic acid, 8.8 mg.; folic acid, 137.8 mcg.; vitamin B_{12}, 8.8 mcg.; choline chloride, 496 mg.; ethoxyquin, 55 mg.; menadione sodium bisulfite, 1.39 mg.; manganese, 146.05 p.p.m.; zinc, 97 p.p.m.; iodine, 2.76 p.p.m.; iron, 44 p.p.m.; copper, 4.4 p.p.m.; and cobalt, 0.44 p.p.m.

[2] 3,5-dinitro-o-toluomide (Zoamix); Dow Chemical Company, Midland, Michigan.

RESULTS AND DISCUSSION

Rather high dietary silicate levels were used in the first experiment primarily because of the difficulty of distinguishing between true responses and normal biological variation when very small dietary modifications of this nature are employed. Two silicates, kaolins A and calcium bentonite, were fed at both the 5 and 10% dietary levels while kaolin B was fed only at the 5% level because of space limita-

[2] Mistrox 60 and Mistrox 47 were furnished by United Sierra, Trenton, New Jersey.

[3] Dixiebond was obtained from International Minerals and Chemical Corporation, Smithville, Mississippi.

TABLE 2.—*The influence of different sources and levels of pellet binding agents on the performance of male chicks (Exp. 1)*

Dietary treatment	Four week results		
	Mean wt., g.[1]	Feed/weight[1]	Prod. cal./kg. meat
Basal	636[ab]	1.58[a]	3,716
Basal+kaolin A, 5%	653[a]	1.66[ab]	3,709
Basal+kaolin B, 5%	624[ab]	1.68[ab]	3,753
Basal+Ca bentonite, 5%	615[b]	1.66[ab]	3,709
Basal+kaolin A, 10%	642[ab]	1.76[bc]	3,725
Basal+Ca bentonite, 10%	562[c]	1.84[c]	3,894

[1] Means within a column and without a common superscript are significantly different ($P<.05$).

tion. Calcium bentonite was the only silicate which significantly influenced growth rate in this experiment (Table 2). At the 10% dietary level, calcium bentonite significantly depressed growth.

Feed utilization was essentially in direct proportion to the energy level of the diet, assuming that the silicates do not contain energy. This point is clearly evident from the column giving the productive Calories required per kilogram of meat produced. The energy required per pound of meat was essentially equal for all treatments, except that the chicks fed calcium bentonite at the 10% level required considerably more Calories per unit of meat than did the other groups. Since this level of bentonite significantly depressed growth, this decreased caloric efficiency is not surprising.

Since our results with pellet binders in the first battery study were in disagreement with the very favorable reports of Ousterhout (1967) and others with respect to their effect on caloric efficiency, a large floor test was conducted. We certainly acknowledge the difficulty in obtaining accurate feed utilization data with small groups of birds in battery experiments.

Table 3 contains the results from the floor experiment in which the silicates were added to the basal starter diet at the 5% level. As would be expected, the 5 and 8 week data show essentially the same trends. Growth rate, as well as feed efficiency, tended to be reduced by the addition of silicates to the diet. A significant reduction in growth at 5 weeks of age was obtained only with calcium bentonite, but at 8 weeks of age, all three silicates significantly reduced growth rate. This reduction in growth at the 5% dietary level was surprising, especially for the kaolins, since even the 10% level of kaolin A did not significantly depress growth in the previous battery test. One possible explanation for this difference is that the basal diets used in Experiment 2 contained somewhat less energy than did the basal diet used in Experiment 1. Growth depression as a result of energy dilution is less apt to occur with high energy diets.

Feed efficiency was also significantly reduced with all three silicates in the floor experiment at both 5 and 8 weeks of age. Bentonite adversely affected feed utilization as well as growth more severely than did the kaolins. This source of calcium bentonite is known to contain a high level of iron (4%) and magnesium (3%)

TABLE 3.—*The influence of different pellet binding agents on the performance of broilers (Exp. 2)*

Dietary treatment	Mean wt.[1], g.		Feed/weight[1]		Prod. cal./kg. meat	
	5 wk.	8 wk.	5 wk.	8 wk.	5 wk.	8 wk.
Basal	830[a]	1,524[a]	1.88[a]	2.26[a]	4,286	5,240
Basal+kaolin A, 5%	812[ab]	1,497[b]	1.98[b]	2.40[b]	4,288	5,289
Basal+kaolin B, 5%	816[a]	1,497[b]	1.96[b]	2.30[b]	4,244	5,267
Basal+Ca bentonite, 5%	771[b]	1,433[c]	2.04[c]	2.49[c]	4,418	5,487

[1] Means within a column and without a common superscript are significantly different ($P<.05$).

which might be the cause for some of the detrimental effects of this silicate. The kaolin contains much less iron and magnesium in comparison.

With the exception of the results obtained with calcium bentonite, which slightly depressed caloric efficiency, the silicates did not appear to exert any influence on caloric efficiency in the floor experiment.

Diets containing all three silicates at the 1, 2 and 3% levels were fed during Experiment 3 (Table 4). It should be noted that the basal diet used in Experiment 1 was also employed in this experiment. Growth rate tended to be improved by the dietary addition of each silicate at either the 1 or 2% dietary level; however, this improvement reached statistical significance only with the 2% level of kaolin A. If the trend toward improved growth rate was real, the optimal level would appear to be between 1 and 2% since the 3% level did not improve growth rate. Caloric efficiency followed the same pattern as growth rate, with the exception of the chicks fed the diets containing 3% kaolin B which exhibited unusually good caloric efficiency. These results, insofar as efficacy levels are concerned, are in agreement with the findings of Almquist *et al.* (1967) who also reported that the 2% dietary level appeared to be optimal. He reported that 2% sodium or calcium bentonite improved the overall nutrient retention by about 3.5%. Our data with the kaolins and calcium bentonite are not nearly so favorable. The only trend toward improved nutrient utilization which we observed was obtained in Experiment 3 with low dietary levels. Small groups in batteries were employed in this experiment and the small differences could be due to biological variation.

TABLE 4.—*The influence of source and levels of pellet binders on the performance of male chicks (Exp 3)*

Dietary treatment	Four week results		
	Mean wt., g.[1]	Feed/ weight[1]	Prod. cal./ kg. meat
Basal	632[a]	1.58[ab]	3,716
Basal + kaolin A, 1%	658[abc]	1.56[a]	3,630
Basal + kaolin A, 2%	681[c]	1.60[abc]	3,683
Basal + kaolin A, 3%	632[a]	1.63[bc]	3,718
Basal + kaolin B, 1%	672[bc]	1.58[ab]	3,678
Basal + kaolin B, 2%	642[ab]	1.60[abc]	3,683
Basal + kaolin B, 3%	636[a]	1.59[ab]	3,628
Basal + Ca bentonite, 1%	662[abc]	1.58[ab]	3,678
Basal + Ca bentonite, 2%	657[abc]	1.58[ab]	3,637
Basal + Ca bentonite, 3%	636[a]	1.65[c]	3,764

[1] Means within a column and without a common superscript are significantly different (P<.05).

SUMMARY

Three experiments were conducted with three silicates, kaolins A and B and calcium bentonite, as dietary variables in broiler diets. Rather high dietary levels, 5 to 10%, were employed in the first two experiments as a dilution to the total diet. At these dietary levels, the silicates tended to depress growth rate with no apparent effect on caloric efficiency, indicating that they are not sources of energy and do not affect the utilization of the dietary nutrients in any way.

When the silicates were tested at lower dietary levels (1–3%), performance tended to be improved at the 1 and 2% dietary levels, but not at the 3% dietary level. However, the results were not consistent. The data from these experiments indicate that the kaolins did not exert any appreciable influence on dietary caloric efficiency. High dietary levels of calcium bentonite, 5 or 10%, depressed chick performance and dietary caloric efficiency.

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THE INTESTINAL RESPONSE TO HIGH BULK FEEDING IN THE RAT

R. H. DOWLING, E. O. RIECKEN, J. W. LAWS AND C. C. BOOTH

Departments of Medicine, Pathology and Radiology,
Postgraduate Medical School, London

(*Received* 20 *December* 1965)

SUMMARY

1. To study the effect of increased dietary bulk on the intestinal tract, groups of rats (twenty in each group) were given diets containing 66% and 80% by weight of powdered kaolin.

2. The animals responded by eating a greater volume of diet, but there was a net reduction in the nutrient value of the food.

3. Barium studies of the gastrointestinal tract in life showed no dilatation or hypertrophy of the small intestine. However, there was radiological dilatation of the caecum in the bulk-fed rats and the weight of the large bowel was significantly greater than in control animals.

4. Histological studies showed that the increase in colon weight was predominantly due to an increased thickness of the muscle coat, and confirmed that there was no small bowel hypertrophy.

5. After bulk feeding, the jejunum showed a marked increase in absorptive capacity of glucose and water.

There is a difference in the anatomical response of the small intestine of the rat following resection of either jejunum or ileum. When the jejunum is resected there is enlargement and hypertrophy of the ileal remnant; on the other hand, there is little or no hypertrophy of a jejunal remnant after removal of the distal small intestine (Booth *et al.*, 1959). These observations suggest that the intestinal hypertrophy which follows resection of the proximal small intestine is not compensatory for loss of length of the intestine but may be due to a different mechanism. An alternative explanation has been suggested by the observations of Kennedy & McCance (1958) who have reported that rats given a diet diluted with three parts of powdered kaolin show enlargement of the small intestine to four times its normal size, the dilatation of

Correspondence: Dr R. H. Dowling, Postgraduate Medical School, Ducane Road, London, W.12.

the gut being principally caused by the large bulk of kaolin in the intestinal lumen. A similar mechanism might possibly explain the enlargement of the distal small bowel that follows proximal resection, since the volume of unabsorbed chyme delivered to the ileum after such a resection must be vastly greater than under normal circumstances.

This paper describes a further study of the effect of dietary bulk on the gastrointestinal tract of the rat, using diets diluted with powdered kaolin. The anatomy of the intestine was studied radiologically in life and subsequently at autopsy. In addition, the functional response of the small intestine to this régime was assessed by measuring glucose transport using the everted sac technique. Studies of the histochemical response of the jejunal mucosa to bulk feeding were also carried out and have been reported elsewhere (Riecken *et al.*, 1965).

MATERIALS AND METHODS

Animals and diets

A hooded strain of in-bred laboratory rats was used. Three groups of rats with initial body weights of approximately 90 g were fed different diets *ad libitum* as follows:

Control Group: Standard diet of mash (Diet 41 B as supplied by Messrs E. Dixon & Sons, Corn Mead Mills, Ware, London);

1:2 Group: Diet containing one part by weight of standard diet and two parts of powdered kaolin;

1:4 Group: Diet containing one part of standard diet and four parts of powdered kaolin.

Twenty rats in each group were studied for a period of 9–12 months. In addition, other groups of animals were given the diets for shorter periods before being killed, in order to assess the speed of onset of the various changes.

Experimental procedure

During the first few weeks body weight was recorded daily and later weekly. The diets were given in deep containers to prevent scatter and the food residue was weighed each day. The daily faecal weight was also recorded for each group of animals.

Intestinal anatomy

Organ weights. The rats were killed by a blow on the head. Immediately after death, stomach, small intestine and colon were removed, carefully washed clean of adherent kaolin, and the fat and mesentery were detached. The tissues were blotted dry on filter paper and weighed.

Histology. Histological sections were prepared from comparable sites of mid-jejunum and mid-colon in the three groups of rats and were stained with haematoxylin and eosin. Using a calibrated micrometer eyepiece, five observations of villous height, mucosal thickness and muscle thickness were taken from each section and the mean reading was recorded in microns.

Intestinal radiology. Barium follow-through examinations were carried out after 9 months of bulk feeding in ten control rats, in eleven animals receiving the 1:2 diet, and in twelve on the 1:4 diet. Each rat was starved for 12 hr and 5·0 ml of barium sulphate suspension (Micropaque) was then introduced into the stomach through a fine polythene tube. A 10×8 in. film cassette was placed in a loose-fitting polythene bag and was positioned for exposure. The rat was placed on the cassette inside the polythene bag which served to immobilize the animal

during the exposure. Films were taken at intervals of 15–20 min for the first hour and at 40–50 min thereafter, until the barium had reached the colon. Radiographic factors were 48 kV, 400 mA, of 0·01 sec at 36 in. anode film distance, using fast film and screens but without a grid.

The radiographic anatomy of the small intestine was studied and the time taken for the barium to pass from the stomach to the colon ('intestinal transit time') was noted. The calibre of the small intestine was taken as the mean diameter of the four widest loops on the films. Measurements were made to the nearest 0·5 mm, no allowance being made for the very slight magnification resulting from the technique. The size of the colon was classified as normal (+), moderately enlarged (+ +), or very large (+ + +).

Intestinal function

Glucose transport. Glucose transport by the first part of the jejunum, distal to the ligament of Trietz, was studied by a modification of the everted sac technique of Wilson & Wiseman (1954). The rats were killed by a blow on the head and four sacs made from each. Both mucosal fluid (0·5–1·0 ml) and serosal fluid (5–10 ml) were Ringer bicarbonate solutions (Krebs & Henseleit modification, 1932) containing glucose 380 mg/100 ml (21 mM), equilibrated with 95% O_2 and 5% CO_2. Sacs were incubated at 37° for 1 hr shaking 80 times/min. Immediately after incubation the sac was gently blotted on moistened filter paper and weighed on a spring balance before draining the serosal contents. The empty sac was then blotted again and re-weighed. The difference between these two weighings gave the final serosal volume. The net serosal fluid transfer was then obtained by subtracting the initial from the final serosal volume. The sacs were dried to constant weight at 170°. After incubation, glucose concentrations were estimated in samples of the serosal and mucosal fluids by glucose oxidase (Huggett & Nixon, 1957). Serosal glucose transport was calculated from the initial and final serosal glucose concentrations and the initial and final serosal volumes, and expressed as mg/100 mg of dry tissue/hr. The mean amount of glucose or fluid transported by the four sacs from each rat was then determined and statistical analysis applied to the mean result for each rat.

Histochemical studies. The enzyme activities of the small intestinal mucosa were studied using the methods described by Pearse (1960). The results of these studies are reported in detail elsewhere (Riecken *et al.*, 1965).

RESULTS

Growth rates and their relationship to dietary intake

The dietary intakes, body weights and the faecal weights in the three groups of animals are shown in Table 1.

The rats whose diet was diluted with powdered kaolin grew less well than the control animals despite a marked increase in the weight of the diet consumed; there was a corresponding increase in the stool weight as a result of the excretion of the unabsorbed kaolin. From inspection of Table 1, there is a discrepancy between the amount of diet eaten by the bulk fed rats and the weight of faeces which they excrete. This is explained partly because evaporation reduces the weight of the food residue thus giving an erroneously high food intake and partly because of incomplete stool collection due to scatter of faecal pellets. Compared with the control group, the animals fed the diet diluted with two parts of powdered kaolin (1:2 diet) ate approximately three times the weight of the control diet and those fed the diet diluted

TABLE 1. Body weight, food intake and stool weights of the three dietary groups (twenty rats in each group) at different time intervals after bulk feeding

Duration of diet (weeks)	Controls				1:2 kaolin				1:4 kaolin			
	Body weight (g)		Mean food intake (g/rat/day)	Mean stool weight (g/rat/day)	Body weight (g)		Mean food intake (g/rat/day)	Mean stool weight (g/rat/day)	Body weight (g)		Mean food intake (g/rat/day)	Mean stool weight (g/rat/day)
	Mean	±SEM			Mean	±SEM			Mean	±SEM		
0	96·6	–	–	–	96·2	–	–	–	95·3	–	–	–
½	117	6·0	26·0	6·8	104	5·7	63·0	24·9	85	5·0	63·0	23·9
1	124	6·2	26·6	6·3	115	6·2	74·9	36·8	100	4·9	81·0	42·9
2	145	6·1	27·0	6·3	131	6·5	75·5	42·4	108	5·2	107·3	61·1
3	151	6·4	25·9	6·0	143	4·5	77·7	41·7	122	5·3	108·0	64·7
4	163	6·3	23·2	6·1	152	4·5	77·1	39·2	125	5·2	101·5	62·7
5	177	5·6	28·8	6·6	160	4·7	77·8	45·4	130	4·5	109·9	63·5
6	174	5·6	25·5	7·0	159	4·5	73·2	42·2	135	4·4	81·8	56·2
7	183	6·1	25·3	8·2	166	4·1	76·7	48·9	130	4·7	91·6	58·5
13	202	5·6	25·0	8·1	183	4·6	66·6	45·2	160	4·3	88·9	77·2
17	209	6·2	25·0	8·1	188	5·5	77·8	49·2	160	4·3	96·2	64·5
21	223	7·1	30·0	8·9	200	4·7	75·5	48·9	175	4·7	101·1	77·2
26	224	6·5	25·8	10·0	183	6·2	77·8	52·8	155	4·3	106·1	68·2
30	239	6·8	32·5	12·3	206	4·5	92·3	58·9	164	4·9	93·3	70·0
34	237	5·1	30·0	7·3	214	3·6	99·4	42·0	183	4·7	98·5	79·0
39	251	7·7	26·0	6·0	220	4·3	78·5	70·6	190	4·0	111·6	92·3

with four parts of kaolin (1:4 diet) ate four times the weight consumed by the controls. The hyperphagia in the animals fed the 1:2 diet developed as soon as the diet was started; in the 1:4 group a similar degree of hyperphagia occurred within the first week of the diet, but this

TABLE 2. Weights of stomach, small intestine and colon at different time intervals after bulk feeding

Duration of diet (weeks)	No. of rats	Dietary group	Stomach (g)		Small intestine (g)		Caecum and colon (g)	
			Mean	±SEM	Mean	±SEM	Mean	±SEM
1–2	3	Control	0·88	0·01	2·95	0·13	1·92	0·12
		1:2 kaolin	0·73	0·03	2·51	0·05	1·82	0·15
		1:4 kaolin	0·78	0·02	3·15	0·09	2·06	0·15
3	3	Control	1·01	0·05	3·70	0·70	2·13	0·27
		1:2 kaolin	0·94	0·05	2·97	0·34	2·54	0·16
		1:4 kaolin	1·00	0·03	3·33	0·66	2·48	0·06
4	4	Control	0·87	0·05	3·40	0·74	1·96	0·12
		1:2 kaolin	0·93	0·04	3·88	0·47	2·88	0·06
		1:4 kaolin	0·93	0·09	4·33	0·61	2·56	0·21
40	16	Control	1·42	0·04	5·04	0·74	2·81	0·09
		1:2 kaolin	1·20	0·02	4·93	0·57	3·79*	0·08
		1:4 kaolin	1·28	0·05	4·99	0·58	4·09**	0·20

* 1:2 group colon heavier than control group colon ($t = 3·24$, $P<0·01$).
** 1:4 group colon heavier than control group colon ($t = 5·03$, $P<0·001$).

TABLE 3. Histological measurements of the small and large intestine after 9 months of high bulk feeding

Dietary group	No. of observations	Small intestine				Large intestine			
		Villous height (μ)		Total mucosal thickness (μ)		Muscle thickness (μ)		Colon muscle thickness (μ)	
		Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM
Control	20	387	17·6	580	9·2	79	4·2	178	11·9
1:2 kaolin	20	400	13·8	500	16·0	77	1·1	318*	11·1
1:4 kaolin	20	4·5	10·4	575	11·2	85	4·1	422**	18·1

* 1:2 group colon thicker than control group colon ($t = 8·6$, $P<0·001$).
** 1:4 group colon thicker than control group colon ($t = 112·5$, $P<0·001$); 1:4 group colon thicker than 1:2 group colon ($t = 4·9$, $P<0·001$).

hyperphagia continued to increase for a further 2–3 weeks before reaching a maximum level. The delay in development of maximum hyperphagia in the group fed the 1:4 diet was associated with a fall in weight during the first week on the diet, whereas the body weights of the animals in the other groups increased steadily.

Anatomical changes in the gastrointestinal tract

The weights of the stomach, small intestine and colon of animals killed at varying time intervals after receiving the control or test diets are shown in Table 2; the results of histological studies of the small and large intestine of the rats killed at 9 months are shown in Table 3.

Stomach. There was no difference in the weights of the stomach from the three dietary groups.

Small intestine. After the 9 months experimental period, the mean weight of the small intestine of the control animals was 5·04 (range 2·81–7·34 g) and there was no significant difference in the small intestinal weights in the bulk fed animals. Histological studies also showed no difference between the three groups when mucosal thickness, villous height or muscle thickness were measured (Table 3).

TABLE 4. Intestinal radiology after 9 months of bulk feeding

Diet	No. of rats	Diameter of small intestine (mm)		Intestinal transit time (min) (mean)	Colon size
		Mean	±SEM		
Normal control diet	10	3·8	0·11	137	+
2 parts kaolin 1 part normal diet	11	4·0	0·15	137	++
4 parts kaolin 1 part normal diet	12	4·0	0·10	139	+++

Colon. After 9 months on the diet, there was a marked increase in the weight of the caecum and colon in both groups of bulk-fed animals. Whereas the mean colon weight in the control rats was 2·81 g (range 2·0–3·45 g), it was 3·79 g (range 3·05–4·29 g) in the animals given the 1:2 diet and 4·09 g (range 2·85–6·09 g) in the 1:4 diet group. Histological studies demonstrated that this increase in colon weight was predominantly due to an increase in the thickness of the muscle coat (Table 3).

Serial studies of a small number of animals killed at weekly intervals after starting the diet showed that this increase in colon weight when compared with control rats had developed within 3 weeks in the rats fed either the 1:2 or the 1:4 diet.

Intestinal radiology

Radiological studies were carried out after 9 months in the control animals and in the bulk-fed rats. The small bowel diameter, transit time, and colon size are shown in Table 4. There was no significant difference in small intestinal calibre or transit time between the three groups of rats (Table 4). However, the caecum of the animals fed the high bulk diets was markedly enlarged when compared with controls.

Functional changes in the small intestine

Glucose transport. The results of the transport studies using the everted sac technique in the rats killed after 9 months on the test diets are given in Fig. 1.

After 9 months of bulk feeding the mean glucose transport by the control rats was

2·52 mg/100 mg dried intestinal tissue (±SEM 0·66) whereas it was 4·81 mg (±0·99) by the 1:2 group and 9·96 mg (±0·53) by the 1:4 group (Fig. 1). The difference in glucose transport between the 1:2 and the 1:4 groups was statistically significant ($t = 4{\cdot}956$, $P<0{\cdot}001$). The difference between the transport in the control group and in the 1:4 kaolin fed rats was also highly significant ($t = 8{\cdot}793$, $P<0{\cdot}001$).

Fluid transfer. The volume of net serosal fluid transfer is illustrated in Fig. 2. The mean fluid transfer for the control group was 0·10 ml/100 mg dry weight intestine (±SEM 0·028), and 0·25 ml (±0·04) in the 1:2 group whilst the 1:4 group transported 0·63 ml (±0·102). The fluid transfer by the 1:4 group was significantly greater than the 1:2 group ($t = 3{\cdot}48$, $P<0{\cdot}01$) and the control group ($t = 5{\cdot}06$, $P<0{\cdot}001$). The 1:2 group also transported more 'water' than the control group ($t = 3{\cdot}09$, $P<0{\cdot}01$).

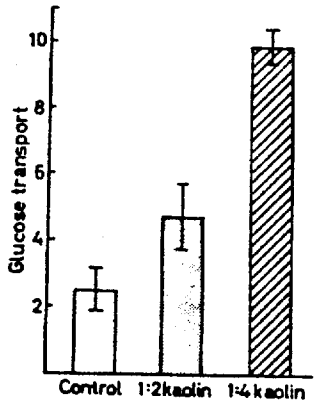

FIG. 1

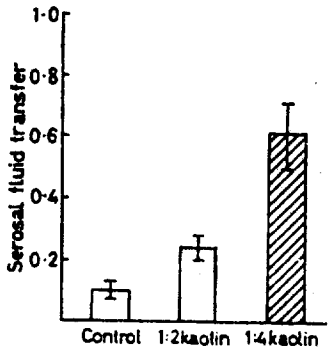

FIG. 2

FIG. 1. Mean glucose transport (mg/100 mg dried intestinal tissue/hr ± SEM) from twelve rats (forty-one sacs) in each dietary group after 9 months of bulk feeding.

FIG. 2. Mean serosal fluid transfer (ml of water transported/100 mg dried intestinal tissue/hr ± SEM) from twelve rats (forty-one sacs) in each group after 9 months of dietary stress.

Histochemical findings. The histochemical enzyme pattern of the small bowel mucosa in the bulk-fed rats showed an increase in the activity of leucine aminopeptidase and to a lesser extent an increase in glutamate and alcohol dehydrogenases when compared with the mucosa from control animals These changes were most marked during the first month of dietary stress (Riecken *et al.*, 1965).

DISCUSSION

The results reported in this paper confirm that animals subjected to the stress of bulk feeding compensate by eating a greater volume of diet than control animals (Addis, 1932; Adolph, 1947; Bohman, Kidwell & McCormick, 1953). Rats in the 1:2 group, for example, ate approximately three times the weight of the diet taken by the controls; their nutrient intake was therefore similar to that in the control animals and it is surprising that they did not grow as well. In those fed the 1:4 diet, however, although the increased alimentation was more marked than in the 1:2 group, it was insufficient to compensate for the dilution of the diet and the nutrient value was therefore less than in the control animals. These observations are similar to those

reported by Kennedy (1950), who found in the rat that increased alimentation compensated for dilution of the diet when the kaolin content was 25% or 50%, but the nutrient value was reduced when the food contained 75% kaolin.

The anatomical response of the gastrointestinal tract to bulk feeding has been studied by previous workers but the results have been conflicting. The intestine has been reported to show either reduction in size (Wetzal, 1928, quoted by Wierda, 1950; Fischer, 1957; Friedman, 1953) or enlargement of either small or large intestine, or both (Addis, 1932; Kennedy, 1950; Friedman, 1953; Fischer, 1957). Our experiments did not show any hypertrophy of the stomach or small intestine after prolonged bulk feeding. When studied radiologically in life, there was no increase in small intestinal diameter, and at autopsy the small intestinal weights of the animals fed the diets diluted with kaolin were not significantly different from the control animals. In addition, histological studies showed that there had been no alteration in the height of the villi, mucosal thickness and the thickness of the muscle coat of the small bowel.

In contrast there was a striking increase in the weight of the colon in the bulk-fed animals (Table 2), the increase being most marked in those given the 1:4 kaolin diet. This increase in colon weight was shown on histological section to be due predominantly to an increase in the muscle coat. This presumably represents a work hypertrophy due to the vastly increased amount of faeces excreted by the bulk-fed animals (Table 1).

The effect of bulk feeding on intestinal absorptive function had not hitherto been studied. Our results demonstrate that although there is no hypertrophy of the small intestine, there is a marked increase in its capacity to transport glucose. The absence of hypertrophy of the small intestine indicates that this increase in transport must be due to an increase in the functional capacity of the intestinal mucosal cells. The histochemical studies indicate that there is an increase in the concentration of enzymes such as leucine aminopeptidase and glutamate dehydrogenase, in the mucosal cells, and it is possible that the increase in intestinal transport is due to a comparable increase in the mechanisms responsible for transport.

The way in which this increase in transport capacity develops is uncertain. Increase of absorptive capacity has been described, however, in response to other types of dietary stress. After 6 weeks of intermittent starvation in the rat, enhanced absorption of glucose and fat have been reported without hypertrophy of the small intestine (Fábry & Kujalová, 1960). Kershaw, Neame & Wiseman (1960) have also demonstrated increased absorption of glucose and L-histidine after as little as 5–9 days semi-starvation in the same species. These changes may be accompanied histochemically by increase in the activity of intestinal mucosal enzymes (Lojda & Fábry, 1959). It is therefore possible that the increased absorptive capacity of the small intestine after bulk feeding, and the histochemical changes, may represent a similar response to a condition of chronic malnutrition.

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Clay- and Cornstarch-Eating Women[1]

CECILE HOOVER EDWARDS, Ph.D.,
SOLONA McDONALD,
JOSEPH R. MITCHELL, M.D.,
LUCY JONES, LOIS MASON,
ALTA MAE KEMP, DORIS LAING,
and LOUISE TRIGG

School of Home Economics,
The Carver Foundation, and
John A. Andrew Memorial Hospital,
Tuskegee Institute, Alabama

THOUGH PRIMITIVE man appears not to have given much thought to nutrition, throughout the centuries man has exhibited foresight in providing for himself a food supply. Literature reports concerning the sociology of diet reveal a growing tendency to consider food as an indicator of cultural values and social processes. Especially in small societies away from urban centers, food choices reflect a deeper integration with symbol and value systems, since food-eating activities form the largest part of human endeavor.

An excellent review of the early literature by Cooper on the subject of pica has recently appeared (1). Hart and co-workers (2), Dickens and Ford (3), Hertz (4), and Whiting (5) have referred to the practices of clay- and cornstarch-eating. Hertz advanced the hypothesis that these practices have their origin in an unconscious endeavor to compensate for certain nutritional deficiences. She suggested that both clay and starch satisfy individual needs, varying from the desire to satisfy hunger to the pleasure of chewing and the desire for social approval. Dickens concluded, in addition, that dirt-eating is a cultural trait, the origin of which is lost. She presented the hypothesis that dirt-eating may be related to hookworm disease, suggesting that the host eats dirt in order to replace the blood constituents removed by the parasites.

Hart demonstrated the ability of pigs to correct severe anemia when they were farrowed out of doors in contrast to being kept indoors on a concrete floor while receiving sow's milk alone.

Edwards, McSwain, and Haire (6) reported the incidence of odd dietary practices revealed by a study of 211 women in Alabama and Georgia made by forty-seven health agencies and ninety-one persons engaged in health work in the southeastern section of the United States. Superstition and tradition appeared to influence the unusual food choices of a large number of women. These workers observed that attitudes and food practices seemed more unusual in rural areas than in or near cities. Older individuals in these rural communities seemed to exert a strong influence on the food choices of the younger women. The ingestion of clay and cornstarch was frequently found to be a part of the dietary pattern of these women, particularly when pregnant.

Since no studies describing the biochemical effects of ingestion of clay and cornstarch have been reported, the authors became interested in pursuing the present investigation. The present article describes the socio-economic backgrounds of the patients, nutritive values of the usual diets of clay- and cornstarch-eaters, and information revealed by the women about these practices. Subsequent articles will describe relationships of clay and cornstarch ingestion to medical and biochemical findings from analyses of blood and excreta of a selected sample of these women, condition of infants at birth, and complications during pregnancy.

Experimental Procedures

The eight-six women who participated in the study were selected from patients at the prenatal and postpartum clinics at the John A. Andrew Memorial Hospital, Tuskegee Institute, Alabama, and from

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[2]Present address: The Agricultural and Technical College of North Carolina, Greensboro.

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previous records of the dietary habits of women. They were divided into six groups: (a) pregnant clay-eaters, (b) non-pregnant clay-eaters, (c) pregnant cornstarch-eaters, (d) non-pregnant cornstarch-eaters, (e) pregnant women who ate neither cornstarch nor clay, and (f) non-pregnant women who did not ingest these unusual substances. The fifth and sixth groups served as controls.

Information about usual dietary patterns was obtained by nutritionists or dietitians, usually at two or three different interviews with each subject. These data were averaged for each woman and tabulated by groups. Medical examinations were made by the obstetrician during periods when the unusual dietary substance was ingested and when it was omitted from the diet. At delivery, information on the mother and infant was recorded.

The research team was composed of a nutritionist, a dietitian, an obstetrician, a clinical laboratory technician, a food chemist, a medical record librarian, a medical social worker, and two graduate students. Public health nurses assisted in making contacts with patients and in follow-up work.

Results and Discussion

Forty-two of the subjects were clay-eaters, twenty-three were cornstarch-eaters, and six ate both bizarre substances. Twenty-one women served as controls. Socio-economic histories revealed interesting information about the subjects' backgrounds (Table 1).

The average age of the women was twenty-eight years, with a range of from fifteen to forty-five years. Seventy-four were married; twelve were single. The number of children in the families of the women averaged 3.8, and ranged from none to 11. Sixty-eight were homemakers, 13 farmed as an occupation, and the remainder held positions as cooks or were engaged in similar occupations.

The income of the group averaged $39 a week, and of this amount, about $13 went for food. Sixty per cent had gardens. Though the average formal educational level was eighth grade, some of the subjects had gone only as far as the second grade. One had begun the senior year of college.

INFORMATION ON CLAY CONSUMPTION

Clay was eaten at any time of the day. Many women consumed it as a part of the menu, stating that the meal didn't seem complete without it. Sixteen ate the substance after meals, and three ate it only before breakfast. Thirty-five experienced no effect of clay-eating on their appetite for food, but five had no desire for food afterward. One woman always ate it before each meal and stated that she had no appetite for food unless she ate clay.

The quantity of clay consumed per day ranged from about 6 to 130 gm. Most of the women obtained the clay from a river or stream bank near their homes. Although interviews revealed that there was no expense connected with obtaining the samples, the authors are aware of the fact that in small southern towns, clay *can* be purchased near the bus station and in similar places. Some of the women, a small number, baked the clay in the oven prior to eating it. This was usually done if the substance was still moist.

The usual sample encountered was light grey and free of rocks and foreign material. Sometimes a streak of pink through the clay suggested the presence of iron. The women stated that the clay tasted sour; white clay was chosen in preference to the red because it was smoother.

If they had a desire for clay and could not get it, twenty-five of the forty-two women stated that they were worried and upset. The women gave these replies in addition to the general statements:

"I feel awful, just about crazy when I can't get clay."
"I feel awful, worse than a person with a headache."

Others said they felt "terrible," "troubled," "disgusted," or "irritable." According to Cooper (1),

TABLE 1 **Socio-economic data of subjects**

GROUP	NUMBER OF SUBJECTS	AVERAGE AGE	MARITAL STATUS		EDUCATION	OCCUPATION			AVERAGE NUMBER OF CHILDREN	AVERAGE INCOME	WEEKLY FOOD EXPENDITURE	WEEKLY EXPENDITURE FOR CLAY OR CORNSTARCH
			Married	Single		Homemaking	Farming	Other				
Clay-Eaters												
		yr.			*yr.*							
Pregnant	38	27	31	7	8	29	7	2	3.4	$33	$13	0
Non-pregnant	4	31	4	0	5	2	2	0	5.5	42	12	0
Cornstarch-Eaters												
Pregnant	20	26	18	2	9	16	3	1	4.0	$42	$16	$0.16
Non-pregnant	3	31	2	1	5	3	0	0	4.0	24	15	0.07
Control Subjects												
Pregnant	12	24	11	1	10	12	0	0	2.0	$50	$10	0
Non-pregnant	9	28	8	1	11	6	1	2	4.0	41	12	0

early literature on pica reveals a similar obsession for clay in clay-eaters. Betten in 1687 and Craigin in 1835 reported (7) that pica permits the mind no rest until it is satisfied. Postell, in a recent publication (8), reviewed the anxiety of planters whose slaves, addicted to dirt-eating, showed utmost craftiness and determination in pursuing the habit in spite of punishment and restraint. Some women in the present study told of being whipped as children to break the habit. This was never successful, and their fathers began collecting clean bits of clay for them. Many, however, had always had clay available.

Thirteen of the women reported that they ate more clay when they were upset about something than during normal times. Craigin revealed a similar observation among slaves, who, when depressed from the rigorous treatment of their masters or other causes, developed the habit of dirt-eating (7).

Thirty-six of the clay group told of feeling satisfied after eating clay. Three stated that they felt "bad" sometimes. Two believed that clay helped them through their pregnancies.

When approached as to why they ate the substance, twenty-six told of having a "taste" for it. One woman enjoyed the feel of clay in her mouth and seven liked its odor.

Though twenty-five were at a loss to explain the desire except to say that they "craved" it, others gave more concrete responses. For example, five women stated that clay relieved nausea during pregnancy, three said it prevented vomiting during pregnancy, and two others thought generally that "it helps babies." Others stated that clay (a) relieved dizziness, (b) helped a weak stomach, (c) cured swollen legs, and (d) relieved headaches.

In *Pica*, Cooper (1) has recorded several beliefs about earth-eating reported by Lasch. It was commonly believed in the latter part of the nineteenth century that earth-eating stopped vomiting, was a remedy for constipation, acted specifically against syphillis, and provided for good posture in the fetus. These early superstitious beliefs are similar to the statements of the women in this study. The old superstition that pica was associated with beautiful progeny has prevailed for centuries. Though in this study no apparent physical symptoms could be directly associated with clay-eating, Craigin (7) and Livingston (according to Cooper [1]) describe these as follows: extreme sluggishness, great edema, pallor of the mucous membranes, susceptibility to cold, diminished secretions, thin and watery blood, shortness of breath, haggard appearance, and progressive weakness. Cooper (1) also notes that Volpata, in a study of illnesses which follow pica, lists gastroenteritis, chlorosis, and pellagra. It would have been extremely interesting in these early studies to have had dietary and medical information on clay-eaters who exhibited such symptoms.

Two of the women told of being constipated by clay and used mineral oil or milk of magnesia as a laxative. One substituted flour when she could not get clay.

When questioned as to what differences they observed in children delivered after periods during which clay was eaten, in comparison to those in which clay was not ingested, one woman stated that her child was "very poor." Two women said that the baby had a "heavy white covering at birth."[4] One woman stated that her friends had told her this would happen.

Practically all of the clay-eaters had a "taste" for the substance only during pregnancy, rejecting it after the baby was born. Twenty-nine told of others in their families who also ate clay. However, the ingestion of clay appeared to be a sex-linked habit, since only three of the eighty-six women had even heard of men who ate clay. Two men in the families of the women were reported to eat clay. Cooper (1) reports observations by Hubright in 1562; Boezo in 1638; and Maler, Schrey, and Dehne around 1700 that pica was common in men. However, the greater number of early writings on this subject deal with discussions of the condition in women and children. The authors' present observations suggest that clay-eating occurs much more frequently in women.

INFORMATION ON CORNSTARCH INGESTION

Most of the twenty-three women who ate cornstarch preferred one brand, which they purchased for about 8 cents a box. Starch consumption ranged from 15 gm. to 2 lb. a day. Thomson (10) reported observations on a woman who ate 4 lb. laundry starch in one week.

Contrary to what might be expected, seventeen of the twenty-three cornstarch-eaters stated that this practice did not affect their desire for food. One woman ate the substance in place of a meal; another stated that she had no desire for food after eating cornstarch, but that she ate her next meal. Oddly enough, cornstarch stimulated a desire for food in another woman.

The women told of different effects of eating cornstarch. One felt relaxed and sleepy afterward. Another said it settled her stomach. One pregnant cornstarch-eater believed it relieved nausea, vomiting, and morning sickness. A majority of the pregnant cornstarch-eaters no longer desired the substance after delivery. One woman stated that her baby had a white covering at delivery.[5]

[4]Unpublished observations by Turner (9) reveal that pregnant clay-eaters usually have sandy-like material in the amniotic fluid when the membranes rupture.

[5]Turner (9) has observed that the newborn infants of cornstarch-eaters are more heavily covered than those of other women. He suggests that this white material on the vernix caseoa is actually cornstarch.

When they had a desire for cornstarch and could not obtain it, one-third of the women became "worried and upset." One stated that she became nauseated.

Seven of the twenty-three ate more cornstarch when they were emotionally upset. After eating the substance, fifteen experienced a feeling of satisfaction. One, who ate blue liquid starch while ironing, stated that she didn't feel well afterward.

It was interesting to delve into reasons the women gave for their attraction to cornstarch. Twenty liked the taste; twelve stated that they "craved" cornstarch. Those who liked the taste usually enjoyed the feel of the substance in the mouth. The odor of starch, particularly when they were ironing, appealed to four women.

Ten of the cornstarch-eaters reported that other members of their families ate the substance. Only one woman in the group of eighty-six had knowledge of a man who ate cornstarch.

SUPERSTITIOUS AND FANCIFUL BELIEFS

The powerful influence of emotions on the choice of food has been proved beyond question. Not all dietary practices, however, have their roots in emotions. Some develop because people have superstitious ideas about food. Indeed, the food habits of man are intimately interwoven with his cultural and religious beliefs. Traditional taboos and superstitions have been passed from one generation to the next and seem firmly entrenched in the customs of specific ethnic groups.

A majority, twenty-five of the forty-two clay-eaters and eighteen of the twenty-three cornstarch-eaters, revealed that the practices were their own habits. This is especially interesting in view of an earlier report by Edwards and co-workers (6) that tradition and superstition seemed to have great influence on the choice of clay and cornstarch by women. In this study, a greater number of pregnant women were included.

All of the twenty pregnant clay-eaters who responded to the question of how the practice was started replied that it was their own. Fourteen of the fifteen pregnant cornstarch-eaters gave similar responses. Only one said she ate the substance because someone told her to do so. All three nonpregnant cornstarch-eaters revealed that the practice was begun in response to a desire for the substance.

Although clay-eating was accepted generally in the rural communities in which the women lived, many, over a period of time, had been given various reasons as to why they should not eat the substance. These reasons ranged from such general statements as "clay might hurt you" or "clay-eating is against health" to more specific statements such as clay-eating would (a) cause hard delivery, (b) kill the woman, (c) cause constipation, (d) dry up the blood, (e) cause the child to "lump up," and (f) cause yellow jaundice, dropsy, tumors, or gas.

On the other hand, strong forces operated in these communities to encourage clay-eating. Several of the women believed that the child would be "marked" if clay were not eaten. Hubright (Cooper [1]) warned in 1562 that frustration of the craving for pica causes an indelible birthmark on the fetus. Cooper in *Pica* (1) also quotes from Zacutus Lusitanius:

> Pregnant patients must be treated gently lest they abort or mark the infant . . . since it is absolutely certain that there is great strength in imagination so that for the most part the color of the thing they imagined is induced in that part of the body which they touched with the hand or fingers at that time, some urge that pregnant women, at the time when malacia arises unexpectedly, should immediately move the hand to the buttocks or legs, thinking it will be more tolerable and wiser that the mark be imprinted upon the buttocks or legs of the fetus than upon its face which is more to be seen and ornamented.

In the present study, while not approving of the habit, many members of the families of clay-eaters felt it better for the woman to eat clay than to crave it and not satisfy the craving.

The responses of the cornstarch-eaters were equally interesting. One woman believed that if she did not consume cornstarch, the baby would be marked. Others, as in the case of the clay-eaters, had been told by members of their communities that the eating of cornstarch (a) was not good for them, (b) caused low blood pressure, (c) was harmful to the child, (d) makes one fat, and (e) causes pellagra.

USUAL DIETARY PATTERNS

The nutritive value of the diets usually consumed by seventy-seven women in the study was calculated. These data are presented in Table 2.

The diets were also rated as follows: When the diet contained 100 per cent or more of the recommended allowance (10) for a specific nutrient, it was rated "excellent" in that nutrient. The rating "good" was assigned when intake of the nutrient was 75 to 99 per cent of the recommended allowance. Diets which contained 50 to 74 per cent of the recommended allowance for a nutrient were rated "fair" in that nutrient, and, diets which contained less than 49 per cent of the nutrient were rated "poor." "Excellent," "good," "fair," and "poor" were assigned numerical equivalents of 4, 3, 2, and 1, respectively. Thus, if a diet was excellent in the nine nutrients, it received a numerical score of 36, and so on. Diets falling in numerical ratings between 36 and 27 were classified as "good"; those ranging between 27 and 18, or 18 and 9 were classified as "fair," or "poor," respectively.

In all groups, the average caloric intake was sub-

TABLE 2 **Nutrients in usual daily diets of women who consumed clay or cornstarch**

GROUP	NUMBER OF SUBJECTS	CALORIES	PROTEIN	CALCIUM	IRON	VITAMIN A	THIAMINE	RIBOFLAVIN	NIACIN	ASCORBIC ACID
Clay-Eaters										
			gm.	*mg.*	*mg.*	*I. U.*	*mg.*	*mg.*	*mg.*	*mg.*
Pregnant	36	1191	51	653	8	9082	1.0	1.4	10	71
Non-pregnant	4	1041	58	461	7	774	0.6	0.8	12	7
Cornstarch-Eaters										
Pregnant	13	984	46	621	7	6550	0.9	1.1	8	81
Non-pregnant	3	1119	44	296	7	6930	0.8	1.7	6	26
Control Subjects										
Pregnant	12	1291	67	658	10	9189	1.1	1.5	17	72
Non-pregnant	9	1709	92	536	11	5974	1.2	1.2	21	71

TABLE 3 **Classification of usual diets of women who ate clay and cornstarch**

GROUP	NUMBER OF SUBJECTS	CLASSIFICATION OF DIET			
		Excellent	Good	Fair	Poor
Clay-Eaters					
Pregnant	36	0	4	11	21
Non-pregnant	4	0	0	2	2
Cornstarch-Eaters					
Pregnant	13	0	1	8	4
Non-pregnant	3	0	1	1	1
Control Subjects					
Pregnant	12	0	1	9	2
Non-pregnant	9	0	5	3	1

stantially less than the recommended allowances. Diets of the non-pregnant controls compared more favorably in this respect, while the intake of calories was approximately 50 per cent of recommended levels in other groups. The caloric intake of the pregnant cornstarch-eaters was less than that of any other group.

When the intakes of other nutrients were compared with the recommended allowances, the diets were generally low in calcium. In addition, diets of pregnant clay- and cornstarch-eaters contained substantially less than the recommended amounts of iron, thiamine, and niacin. Control diets rated more favorably in these nutrients. This observation is especially interesting in view of the hypothesis that clay may provide a source of calcium and iron. The average hemoglobin levels of clay-eaters were less (9.0 and 8.8 gm. per 100 ml. for pregnant and non-pregnant groups) than those of controls (10.1 and 9.9 gm. per 100 ml., respectively). The well known relationships of thiamine and niacin to the metabolism of carbohydrate would suggest a greater need for these nutrients in the diets of the cornstarch-eaters. In studies reported by Ferguson and Keaton (11), 94 per cent of the diets of rural pregnant women in Mississippi were inadequate, and hemoglobin levels were unusually low. Forty-one per cent of the 331 Negro women ate cornstarch and 27 per cent ate clay.

The diets of the non-pregnant clay-eaters were very low in ascorbic acid, vitamin A, and thiamine; those of non-pregnant cornstarch-eaters were low in calcium, niacin, and ascorbic acid.

It can be observed from Table 2 that the diets of the control groups supplied more calories, protein, iron, thiamine, riboflavin, niacin, and ascorbic acid than did those of the experimental groups. Cooper (1) cites the work of Renaud who related pica to poor or scanty diets.

These data suggest that the self-selected diets of clay- and cornstarch-eaters may have precipitated a need for additional nutrients which may have been supplied by clay (iron and calcium) and cornstarch (calories).

Eighty-nine per cent of the usual diets of the pregnant clay-eaters and 100 per cent of those of the non-pregnant clay-eaters were rated "fair" to "poor" (Table 3). Only 11 per cent of the diets of the pregnant clay-eaters were rated as "good" and none of those of the non-pregnant clay-eaters fell in this category.

Ninety-two per cent of the diets of the pregnant controls were rated "fair" or "poor," and 44 per cent of the diets of the non-pregnant controls were in these categories. A higher percentage, 56, of the meals of the non-pregnant controls was rated as "good."

Evaluation of the dietary patterns of the experimental and controls groups revealed that breakfast and lunch were the meals most frequently missed. Dinner was eaten six or seven times a week by all groups. The pregnant clay- and cornstarch-eaters consumed an average 2.3 cups milk per day as compared with 3.3 cups in the control pregnant group. Non-pregnant experimental groups consumed 1 cup milk per day in contrast to 2 cups by the control group.

The money spent on food per family per week was approximately the same for all groups and ranged from $10 to $16.

FOOD CHOICES AFTER DELIVERY

The diet chosen most frequently by these women immediately after delivery contained rice, grits, white bread or toast, vegetables, and milk. There appeared to be a tendency to restrict the menu to light, starchy foods, though eggs and chicken were frequently added. If meat were chosen, it was most often pork. Turnips or some type of greens or peas were frequently the vegetable consumed. The majority of the women restricted themselves to this limited menu for a few weeks after birth of the baby.

Summary

The effects of clay and cornstarch ingestion by eighty-six women were studied. Thirty-eight were pregnant clay-eaters, and four were non-pregnant; twenty were pregnant cornstarch-eaters, and 3 were not pregnant. Six women ate both clay and cornstarch. Twelve pregnant women and 9 non-pregnant women served as controls.

The diets of pregnant clay- and cornstarch-eaters were substantially low in calories, calcium, iron, thiamine, and niacin. Fifty-four per cent of the diets of clay-eaters and 32 per cent of the diets of cornstarch-eaters were classified as "poor," whereas only 14 per cent of those of controls were so rated.

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ANALGESIC EFFECTS OF IRRITANTS IN THREE MODELS OF EXPERIMENTALLY-INDUCED PAIN (1)

BY

J. T. HITCHENS, S. GOLDSTEIN, I. SHEMANO AND J. M. BEILER

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The empirical use of irritants for the relief of both pain and inflammation can be traced back many centuries (1). Experiments on human subjects, objectively demonstrating that pain in one part of the body could significantly elevate pain threshold in another part, were reported in 1936 by GAMMON *et al.* (2–4), and in 1937 by DUNCKER (5). Using radiant heat as the algesimetric method, HARDY *et al.* (6) in 1940 demonstrated that tourniquet pain raised the pain threshold of skin as much as 35 per cent. In 1946, PARSONS and GOETZL (7), applying an AC current through metal tooth fillings in human subjects, found that the voltage required to induce pain was markedly increased following topical application of ethyl chloride to a small area on the leg. In 1953, MACARTHUR and ALSTEAD (8) reported that topically applied chemical irritants were effective in relieving the pain induced by i.m. injection of hypertonic saline.

There are many reports of chemical irritants producing anti-inflammatory effects in various animal tests (9–13); however, there is surprisingly little information on the analgesic effects of irritants in experimental animal models. WINTER and FLATAKER (14), using a rat tail pinch method, recently demonstrated antinociceptive effects with formalin (injected into the rat's foot), phenylquinone and stearic acid (injected i.p.).

(1) A preliminary report of this work was presented at the Fall Meeting of the American Society for Pharmacology and Experimental Therapeutics, Philadelphia, Pennsylvania, August 1965.

The objectives of this report were to determine the effects of irritants given by various routes of administration in 3 different models of experimentally induced pain, and to determine whether the analgesic action was due, at least in part, to a humoral factor. Previous reports indicate that irritant-induced anti-inflammatory effects are mediated via a humoral mechanism (10, 13).

Methods and Materials

In the following studies, formalin was administered as an aqueous solution; the concentrations were based on a solution of 37 per cent by weight equivalent to a 100 per cent concentration. Kaolin was administered in a 0.5 per cent gum tragacanth suspension. Croton oil [1] was administered as an aqueous suspension in 10 per cent Tween 80 and distilled water. In all cases, the volume administered was 0.1 ml/10 g of body weight. Results were considered significant when the P value was equal to or less than 0.05.

Rat Pressure Pain Test.

The method was similar to that described by Randall and Selitto (15) with the exception that no inflammation was induced in the rat's foot, and younger male rats, 60 to 80 g were used. Sprague-Dawley CD rats obtained from Charles River Breeding Laboratories were employed. Pressure via a metal peg mounted on the end of a syringe was applied to the plantar surface of the non-inflamed foot at the rate of 20 mm Hg per second. Pain threshold was reached when the rat struggled. The normal control threshold response occurred at approximately 30 mm Hg. The mean of two control responses was compared to the maximal response obtained following administration of the test agent and the results were expressed as per cent change in pain threshold. A vehicle-treated control group was tested concurrently. Statistical significance of drug changes was determined using Student's "*t*" test.

Phenylquinone Writhing Test.

This procedure was a modification of that reported by Hendershot and Forsaith (16). Male albino CD mice (18 to 24 g) from Charles River Breeding Laboratories were used. The writhing response was induced by an i.p. injection of 0.25 ml of a 0.02 per cent solution of phenylquinone. Protection against the writhing syndrome was an indication of analgesia. Ten mice were used per dose and ED_{50}'s and 95 per cent confidence limits were established by the Litchfield-Wilcoxon method (17).

Mouse Hot Plate Test.

This procedure was a modification of that described by Eddy and Leimbach (18) and measures the reaction time of mice dropped onto a copper plate maintained at 55° C. The reaction criteria were licking of the hind feet or attempting to jump out of a restraining cylinder. Two control reaction times were determined for each animal and the animals were tested at various intervals following administration of the test materials. Analgesia was expressed as maximum per cent increase in reaction time relative to the pretreatment values. Statistical significance was determined using Student's "*t*" test.

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[1] Croton oil N.F. VII, Magnus, Mabee and Reynard Inc., New York, New York, U.S.A.

TABLE I

Pressure pain test in rats (1): Per cent increase in pain threshold following administration of irritants by various routes

Treatment	% Concentration of Irritant	Routes of Administration: Intraperitoneal		Subcutaneous		Oral	
		No. of Rats	% Increase in Pain Threshold ± S.D.	No. of Rats	% Increase in Pain Threshold ± S.D.	No. of Rats	% Increase in Pain Threshold ± S.D.
Kaolin	Controls	8	18.0 ± 8.7	16	9.5 ± 4.2	16	10.3 ± 9.7
	0.0625	8	102.3 ± 20.4 (2)				
	0.25	8	105.3 ± 40.0 (2)				
	1	8	132.0 ± 25.1 (2)				
	2			8	32.7 ± 21.0 (2)	8	18.8 ± 16.2
	4	8	125.8 ± 45.4 (2)	8	47.1 ± 38.6 (2)	8	33.9 ± 25.7 (2)
	8			8	46.3 ± 29.3 (2)	8	35.2 ± 29.0 (2)
	16			16	102.4 ± 46.4 (2)	16	52.1 ± 8.5 (2)
Formalin	Controls	8	7.6 ± 6.9	8	10.4 ± 8.2	8	16.3 ± 18.8
	0.25	8	18.9 ± 13.4				
	0.5	8	38.4 ± 27.2 (2)	8	35.5 ± 15.2 (2)		
	1	8	76.8 ± 46.4 (2)	8	43.9 ± 22.6 (2)	8	67.9 ± 41.3 (2)
	2	8	132.8 ± 27.3 (2)	8	65.7 ± 27.9 (2)	8	44.1 ± 25.9
	4			8	129.2 ± 26.9 (2)	8	99.0 ± 46.0 (2)
	8					8	121.5 ± 19.9 (2)

(1) = Sprague Dawley CD rats. (2) = Values represent significant ($P \leq .05$) change from controls.

TABLE I (Continued)

Pressure pain test in rats (1): Per cent increase in pain threshold following administration of irritants by various routes

Treatment	% Concentration of Irritant	Routes of Administration: Intraperitoneal		Subcutaneous		Oral	
		No. of Rats	% Increase in Pain Threshold ± S.D.	No. of Rats	% Increase in Pain Threshold ± S.D.	No. of Rats	% Increase in Pain Threshold ± S.D.
Croton Oil	Controls	24	13.8 ± 9.2	8	19.9 ± 6.9	7	14.1 ± 5.6
	0.0000625	16	17.4 ± 11.2				
	0.00025	16	48.8 ± 25.8 (2)				
	0.001	24	95.6 ± 36.3 (2)				
	0.004	24	110.3 ± 36.2 (2)				
	0.008			8	70.0 ± 34.4 (2)		
	0.031			8	53.8 ± 32.3 (2)		
	0.125			8	81.1 ± 43.4 (2)		
	0.2					8	60.9 ± 27.7 (2)
	0.5			8	100.8 ± 54.0 (2)		
	1					8	70.0 ± 31.6 (2)
	5					8	81.1 ± 28.2 (2)
	25					8	108.4 ± 32.4 (2)
	100						

(1) = Sprague Dawley CD rats. (2) = Values represent significant ($P \leq .05$) change from controls.

Acute Toxicity Studies.

Male albino Sprague-Dawley rats (60 to 80 g) obtained from Charles River Breeding Laboratories were used. Ten rats were employed per dose, and LD_{50}'s were determined after 7 days using the LITCHFIELD-WILCOXON method (17).

Parabiotic Studies.

Male Lewis inbred rats from Microbiological Associates weighing 65 to 85 g were used. The animals were anesthetized with ether and united using the procedure described by BUNSTER and MEYER (19). Circulatory communication between the 2 animals was tested seven days after surgery by injecting Evan's Blue i.v. (0.5 ml of a 1 per cent solution). The only pairs that were used for testing were those in which the second rat turned blue within 90 min following the dye injection. One week later these pairs were used in the pressure pain test. The irritant was injected i.p. into one of the rats; both animals were tested over a 6 hr. period for changes in pain threshold. For comparative purposes, non-parabiotic Lewis rats were also tested.

RESULTS

The effects of kaolin, formalin and croton oil in the rat pressure pain test are summarized in Table I. All 3 irritants, administered by the oral, s.c. or i.p. routes, produced significant elevations in pain threshold. The i.p. route appeared to be the most sensitive, and by this route clear-cut dose responses were obtained. Table II compares the LD_{50} concentrations

TABLE II

Comparison of acute toxicity in rats (LD_{50} concentration) with the lowest effective analgesic concentrations tested (pressure pain test)

Treatment	Toxic Concentration / Analgesic Concentration		
	Intraperitoneal	Subcutaneous	Oral
Kaolin	1.7 / 0.0625	> 16 / 2.0	> 8 / 2.0*
Formalin	3.85 / 0.5*	> 4 / 0.5	> 16 / 1.0
Croton Oil	0.082 / 0.00025*	> 2 / 0.008	> 8 / 0.2

* = Minimal effective concentrations.

of the irritants with the lowest effective analgesic concentrations tested. It is evident that there is a considerable separation between toxicity and analgesic effects with all 3 irritants, and by all 3 routes of administration.

TABLE III

Phenylquinone writhing test: Effects of irritants administered by various routes

Treatment	ED_{50} Concentration and 95 Per Cent Confidence Limits		
	Intraperitoneal	Subcutaneous	Oral
Kaolin	1.78 (1.34–2.35)	> 16	> 16
Formalin	1.41 (1.08–1.83)	1.9 (1.12–3.23)	> 8
Croton Oil	0.016 (0.005–0.041)	1.72 (0.96–3.1)	3.55 (2.25–5.61)

TABLE IV

Mouse Hot Plate Test: The Effects of Irritants

Treatment	Route of Administration	Per Cent Concentration of Irritant	No. of Mice	Per Cent Increase in Pain Threshold ± S.D.
Kaolin	i.p.	Control	15	10.0 ± 61.6
		4	15	99.2 ± 62.3 (1)
Formalin	i.p.	Control	16	8.9 ± 41.9
		0.5	8	16.8 ± 34.8
		1	8	87.2 ± 60.2 (1)
		2	15	107.9 ± 78.1 (1)
		4	15	131.9 ± 83.2 (1)
Croton Oil	i.p.	Control	23	14.7 ± 37.5
		0.040	7	143.0 ± 84.3 (1)
		0.079	8	102.8 ± 67.9 (1)
		0.126	16	80.7 ± 102.2 (1)
		0.200	16	67.9 ± 61.5 (1)
		0.316	16	104.7 ± 77.1 (1)
	p.o.	Control	8	3.8 ± 38.7
		1	8	49.7 ± 66.2
		1.6	8	75.0 ± 85.5 (1)
		2.5	8	118.2 ± 89.9 (1)

(1) = Values represent a significant ($P \leq .05$) change from controls.

In the phenylquinone writhing test (TABLE III), croton oil was active by all 3 routes of administration. Formalin, however, was inactive orally up to a concentration of 8 per cent, and kaolin, at concentrations up to 16 per cent, was inactive both p.o. and s.c.

In the mouse hot plate test (TABLE IV) all 3 irritants were shown to

TABLE V

Pressure pain test in rats: Per cent increase in pain threshold following i.p. administration of kaolin to parabiotic (Lewis-inbred) rats

Per Cent Concentration of Kaolin i.p.	No. of Parabiotic Pairs Tested	Per Cent Increase in Pain Threshold ± S.D.	
		Treated Parabionts	Non-Treated Parabionts
Controls (0.5 % Tragacanth)	32	22.8 ± 26.1	12.9 ± 19.7
4	29	57.9 ± 40.3 (1)	16.3 ± 20.7
8	9	66.5 ± 38.3 (1)	5.6 ± 15.5
16	9	77.3 ± 40.6 (1)	10.9 ± 11.9

(1) = Values represent a significant ($P \leq .001$) difference from both the non-treated rats and the control group.

TABLE VI

Pressure pain test in rats: Per cent increase in pain threshold following i.p. administration of kaolin to Lewis-inbred rats

Per Cent Concentration of Kaolin i.p.	No. of Rats Tested	Per Cent Increase in Pain Threshold ± S.D.
Controls (0.5 % Tragacanth)	20	9.43 ± 14.1
4	20	50.8 ± 33.2 (1)
8	8	65.5 ± 43.8 (1)
16	8	112.3 ± 41.5 (1)

(1) = Values represent a significant ($P \leq .001$) change from controls

produce significant analgesic effects by the i.p. route. Croton oil was tested p.o. and also found to produce significant effects.

Table V summarizes the results obtained in the rat pressure pain test following i.p. administration of kaolin to parabiotic (Lewis-inbred) rats. Kaolin, administered in concentrations of 4, 8 and 16 per cent, produced significant analgesic effects in the rats receiving the injection, but had no effects on their attached partners. The degree of analgesia was much less than that previously observed in the Sprague-Dawley rats (TABLE I). The effects of kaolin in the parabiotic rats were, however, similar to those observed in non-parabiotic Lewis rats (TABLE VI), indicating that the 2 strains of rats differed markedly in their analgesic response to injected kaolin.

DISCUSSION

The results of these studies confirm and extend those reported by WINTER and FLATAKER (14). Chemical irritants, such as croton oil, formalin and kaolin, are capable of producing analgesic effects when tested in several different analgesic screening models. These effects can be demonstrated following oral as well as parenteral administration, depending upon the irritant and the test model employed. Independent of the route of administration, the irritants also demonstrated a wide separation between analgesic doses (rat pressure pain test) and doses producing lethality.

The analgesic profile of the irritants appears to be similar to that of the potent, centrally acting analgesics, in that both groups of agents are active in all 3 analgesic models (rat pressure pain test, hot plate test and phenylquinone writhing test). The weaker (aspirin-like) analgesics, however, were inactive in the pressure pain and hot plate tests. Thus it is apparent from these studies that an irritant could easily be mistaken for a potent, centrally acting analgesic, with a wide therapeutic index. Potential analgesic agents should therefore be checked for their tissue irritating properties.

The results of the parabiotic studies in rats indicate that the irritant-induced analgesic action does not appear to be humorally mediated. Several theories have been proposed to explain the action of the irritants in the relief of pain (20), although the exact mechanism still remains obscure.

In contrast to the above findings, the anti-inflammatory action produced by chemical irritants has been previously attributed to a humoral mechanism. LADEN *et al.* (10) demonstrated that blood

obtained from irritant-injected rats reduced a pleural edema when injected i.v. into other rats, and GOLDSTEIN *et al.* (13) demonstrated that kaolin, administered i.p. to one rat of a parabiotic pair, significantly reduced the size of a carrageenin-induced abscess in the attached partner. Chemical irritants, therefore, appear to produce analgesic and anti-inflammatory actions by two different mechanisms.

SUMMARY

The irritants, croton oil, formalin and kaolin were tested for their analgesic effect in 3 different models of experimentally induced pain (a rat pressure pain test, the mouse hot plate and the phenylquinone writhing tests). The results of these studies indicate that irritants are capable of producing analgesic effects by the oral, s.c. or i.p. routes of administration, depending on the test model and the irritant used. Studies using parabiotic rats indicate that the analgesic effects induced by the irritants were not mediated through a humoral mechanism.

ACKNOWLEDGEMENTS

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The hypothalamic control of food intake in rats

By G. C. Kennedy

National Institute for Medical Research, London, N.W. 7

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[Plates 24 and 25]

The effect on feeding behaviour in the rat, of permanent electrolytic lesions in the hypothalamus which cause obesity, has been studied.

Soon after operation, the animals were extremely voracious. As the obesity became established, the voracity was reduced in degree and finally disappeared. Young unoperated rats could not be made to exhibit such voracity even after starvation.

The young unoperated animals showed little discrimination against unattractive diets. When fed on meal mixed with an equal weight of kaolin, they obtained a normal nutrient intake, and maintained their weight by doubling the bulk eaten. In the voracious stage, operated animals adapted in this way more quickly than normal; in the obese stage they adapted more slowly and only after loss of considerable body fat. Older unoperated animals, which were moderately obese, resembled the operated ones in their feeding reactions.

It is suggested that the effect of hypothalamic damage is to emphasize two components of the urge to eat, a primitive voracious one, and a more discriminative component, either of which may be dominant according to the state of the fat stores. The simplicity of the young rats' reactions appears to be due to closer co-ordination of these components by the hypothalamus. Modification of the hypothalamic control of energy intake may be a pre-requisite of obesity in general.

Reasons are advanced for regarding the hypothalamic mechanism as being sensitive to chemical changes in the blood.

Introduction

Obesity has long been known to be associated in some way with the hypothalamic-pituitary complex. Brobeck (1946) attributes the first clinical description of rapidly increasing weight associated with a tumour of this region to Mohr in 1840. Babinski (1900) and Fröhlich (1901) described obesity in adolescents associated with destructive pituitary lesions, and attributed it to diminished secretion of the gland. However, in 1904, Erdheim expressed the dissident view that the source of the metabolic disturbance in Fröhlich's syndrome lay in the hypothalamus, and the aetiology has remained a subject of controversy ever since. The recent experimental studies of adiposity following exclusively hypothalamic damage in animals, notably by Ranson, Brobeck and their co-workers, have done much to simplify the position.

The experimental approach to the 'hypothalamic' theory of obesity may be said to have begun with the work of Camus & Roussy (1913, 1920, 1922) on the dog, and of Smith (1927, 1930) on the rat. These workers showed that removal of the pituitary was not followed by increased adiposity, whether the removal was partial or complete, so long as the base of the brain was not damaged. Where obesity did occur, the hypothalamus was found to be affected. Various workers since then have recorded obesity following injury which appeared to be mainly hypothalamic, although, because of the subtemporal routes of operative approach generally used, damage to the gland could not be excluded with certainty.

In 1939, Hetherington & Ranson, using the Horseley-Clarke stereotaxic instru-

ment, were able to produce obesity in the rat by electrolytic lesions, introducing the electrode downwards through the parietal bone to avoid suspicion of damaging the pituitary. Brobeck, Tepperman & Long (1943) confirmed this work, which has subsequently been extended to the cat and the monkey.

The lesions produced by Ranson were fairly widespread in the ventro-posterior hypothalamus, but the most effective ones always appeared to involve the ventro-medial nuclei, or fibres which Ranson believed to leave their ventro-posterior aspect. Hetherington (1943) showed that hypophysectomy either before or after the hypothalamic operation did not prevent the increase in weight, and concluded, '...since neither total nor partial hypophysectomy produces adiposity, or prevents its appearance after hypothalamic damage is done, it is not likely that the hypophysis is involved in the production of obesity often associated with injury to structures in the pituitary region'.

Brobeck, with rather more discrete lesions, obtained extreme obesity by damaging the same area of the hypothalamus. Investigation showed that in most cases the food intake was increased, sometimes to two or three times normal, and at the same time the spontaneous activity of the animal was reduced. Sometimes the hyperphagia, as Brobeck has called it, appeared to be no more than could be expected from the excess weight of the rat, but in other cases the appetite actually decreased as the rat became fatter until a balance was attained.

There is a marked difference in the relative importance attributed to the intake and activity factors by the two schools of workers. Hetherington & Ranson (1942) record that some of their most obese rats did not show an increased food consumption, but that most of the animals in which they damaged the hypothalamus, whether they became obese or not, showed greatly reduced activity. Brobeck's more localized lesions produced obesity of more dramatic onset, and usually of greater degree, and in his animals hyperphagia was often obvious from the time of recovery from the anaesthetic; there can be little doubt that it was the major cause of obesity. In view of the difference in the extent of the lesions, it is possible that Ranson was dealing with multiple effects, and Brobeck with a single one. Investigation of other factors which might be expected to produce economy of energy, such as changes in intestinal absorption or intermediate metabolism, has shown only minor and inconstant abnormalities, which appear quite inadequate to explain the great rates of weight increase which have been observed. These investigations have been reviewed by Brobeck (1946).

To summarize, it appears that exclusively hypothalamic damage can cause extreme obesity which is adequately explained by the increase of food intake observed during its development. In its most characteristic form, this hypothalamic obesity is not accompanied by any other metabolic change, or decrease in activity, which might significantly influence the 'metabolic equation', nor does the pituitary appear to play any part.

The present investigation has been undertaken to examine more closely the nature of the disturbance in feeding behaviour which Brobeck has called hyperphagia, and to find whether any signs of the activity of a hypothalamic 'centre' can be detected in the normal animal.

Methods

Animals and operative procedure

Initially, two different Wistar strain albino rats were used, one of them differentiated in table 1 by the prefix B, and a hooded strain with the prefix H. Litter-mate controls were always used. The strains appeared to vary in their tendency to become fat under normal conditions, but no difference was detected in their capacity to develop obesity after operation. Later work has therefore been restricted to Wistar strain albinos, indicated by plain numbering, to which all normal growth data quoted refer. The operations were carried out under intraperitoneal bromethol anaesthesia, using a Horseley-Clarke stereotaxic instrument in the manner described by Ranson and Brobeck. The only essential post-operative precaution appeared to be the maintenance of body temperature by warming the room to 75 to 80° F.

Objective estimation of the degree of obesity

Assessment of obesity may be difficult where the rate of skeletal growth of an animal differs much from that of its litter-mate control. The relation of weight to nose-anus length was therefore investigated in the normal animal. Ranson found the relation between weight and the cube of length to be inconstant, so we based our analysis on the more general function expressed by Huxley's allometric growth equation $y = bx^{\alpha}$ with weight and length as the variants y and x. All lengths were determined under anaesthesia. Using data from eighty-three animals measured within the same two weeks, analysis for linear regression of log weight on log length gave a correlation coefficient of 0·9868 with $P < 0{\cdot}01$. The slope of the regression line, which is shown in figure 1, is approximately 3·25. This is consistent with Huxley's equation, expressed in the logarithmic form $\log y = \log b + \alpha \log x$. The slope is sufficiently different from 3 to explain Ranson's difficulty with a simple cubic relation, especially as his published data on control animals yielded a regression line of very similar slope to that of figure 1.

From the length of an obese animal, reference to the curve gives the 'regression weight' of a control of equal length; I have referred to weights calculated for obese animals in this way as 'ideal weights'.

Determination of the fat and water content

Fat and water content of the rats was determined separately on the skin with the adherent subcutaneous tissue, and on as large an aliquot as possible—usually about 50 g.—of the remaining minced carcass. Before mincing, the gut was opened and wiped clean of contents, in order that comparisons with animals on high-residue diets should be valid. The water was first distilled off under reflux with benzene, and collected in an extractor tube arranged to spill back the benzene into the distillation flask. Twelve hours extraction gave a constant volume of water. The dehydrated tissue was then further extracted with the same benzene in a Soxhlet apparatus. Re-extraction with ether was found to give a negligible increase in fat yield. Finally, the residue was dried and weighed.

This method makes it possible to determine fat, water and dried residue fractions directly on the same large sample of tissue. We found the calculated fractions of the carcass as a whole yielded a total which differed little from the body weight directly measured. The mean error in determinations was 2·403 ± 0·224 %.

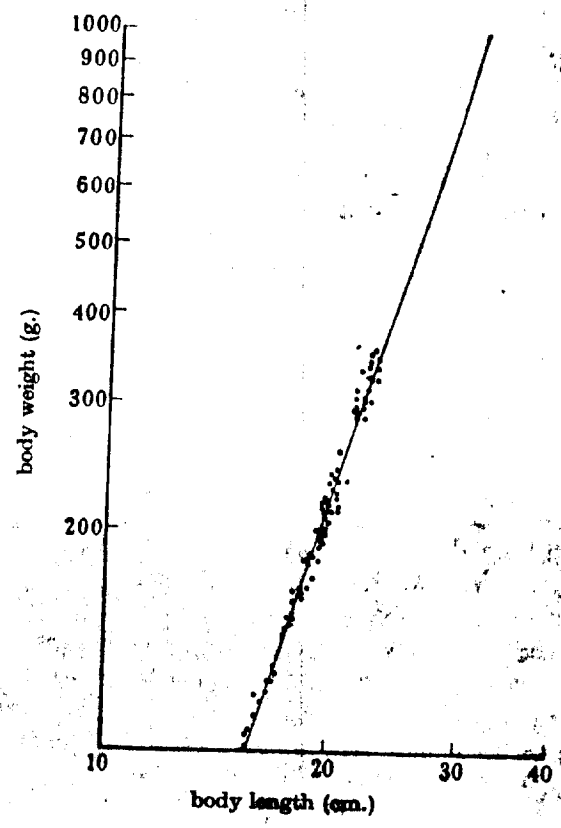

FIGURE 1. Relation between log weight and log length of normal albino rats. Slope = 3·25; $r = 0·9868$; $P < 0·01$.

Feeding of adulterated diets

Adolph (1947) showed that young rats presented with a diet containing varying percentages of an adulterant such as kaolin or cellulose responded within 2 or 3 days by increasing their overall consumption until the amount of nutrient eaten was restored to its original level. His rats were able to make this adjustment until something between 30 and 50 % of diluent had been added to the diet, after which the mechanism apparently failed; he suggested that the limiting factor was gut size.

This technique has been applied to young normal rats of different age groups, and to hyperphagic animals, and marked variations in feeding reactions have been observed. The test diets employed were the stock meal used in this laboratory, powdered finely to prevent selection, and mixtures of this with kaolin containing respectively 75, 50 and 25 % of stock diet. Rats were kept on wire grids over clean

paper to detect spilling, most of which was prevented by placing the food cups inside deep metal containers. All spilt food was collected and weighed. Room temperature was kept between 75 and 80° F.

Results

Position of effective lesions

During the initial stages of the work, in a series of 120 animals, bilaterally symmetrical lesions were placed in as many positions as possible in the hypothalamus and rostral part of the mid-brain. Although these animals were kept for 6 months, none of them showed any sign of becoming obese, except where the lesion was in the area described by Ranson and by Brobeck, in the lateral part of the tuberal region. It has been my experience also that the most effective lesions lie ventro-lateral to the ventro-medial nuclei, and often appear to cause little direct damage to the nuclei themselves. It has been impossible to confirm the finding of Hetherington & Ranson, that obesity could be produced by lesions caudal to these nuclei, even when two large lesions were placed on each side of the mid-line. Figure 2, plate 24, shows the lesions in rat 230, and figure 3, plate 25, the degree of obesity exhibited by the animal compared with its control at autopsy, 19 weeks after operation, illustrating the enormous increase in depot fat seen in these animals.

Analysis of carcasses

The water, fat and dry-residue percentages of ten obese rats and their controls are given in table 1. Comparison is helped by expressing the percentages in terms of the ideal weight, when it is seen that the body water is unaffected by the operation. The dry residue is slightly increased, more markedly in the two fattest animals 130 and 267, but the increase is of a much smaller order than that in neutral fat, and may well be explained by changes in musculature secondary to the greatly increased weight carried. Comparison of the 'ideal weights' with the weights of the controls makes it clear that there is no consistent change in skeletal length, such as might be expected if there were any pituitary disturbance.

In table 2 a comparison is made of three possible methods of estimation of adiposity. The regression method, in which the ideal weight is subtracted from the observed weight, gives a better agreement with the results of fat analysis than direct comparison with the litter-mate control. This is especially marked in the case of animal 173, which was much shorter than its control, and although moderately obese, was almost equal to the control in weight.

The increase of weight in these animals is fully accounted for by excessive fat deposits, and apparently neither water retention nor 'growth' in its usually accepted sense plays any part.

Feeding behaviour on stock diet

Animals with hypothalamic lesions often eat voraciously immediately after recovery from the anaesthetic. Such behaviour was noted by Brobeck, who recorded that some of his animals ate more in the first 24 hr. after operation than in any subsequent day. While this was also our experience, we noted that such voracity

was not a reliable prognostic sign of future obesity, as the increased appetite was often not maintained for more than a few days. On the other hand, the permanent type of hyperphagia was usually recognizable by the end of the first week, when an increase in weight of 50 g. had often occurred, and the daily food intake was from

TABLE 1. ANALYSIS OF OBESE RATS AND THEIR CONTROLS

rat no.	weight (g.)	ideal weight (g.)	percentage water		percentage fat		percentage residue	
			actual	ideal	actual	ideal	actual	ideal
126*o*	397	315	52·2	64·5	30·6	38·6	19·6	24·7
130*o*	516	290	33·4	59·3	48·5	36·5	16·1	28·6
129*c*	318	325	64·4	63·0	12·0	11·7	24·2	23·7
165*o*	304	224	46·8	63·6	35·4	48·0	19·8	26·9
169*c*	215	224	64·6	62·0	14·2	13·7	24·0	23·0
173*o*	376	335	51·25	57·6	30·8	34·6	19·2	21·5
171*c*	372	390	57·6	55	17·2	16·3	20·5	19·5
176*o*	292	224	47·2	61·6	42·0	54·7	18·3	23·8
177*c*	216	224	65·5	63·1	17·5	16·9	23·9	23·0
230*o*	356	185	33·3	64·0	53·5	100·3	13·5	26·0
224*c*	203	210	61·1	59·0	18·2	17·5	22·5	21·8
267*o*	524	285	37	68·1	46·4	85·1	18·4	33·7
266*o*	336	280	52·6	62·9	27·0	32·3	21·2	25·4
268*c*	254	270	63·2	60·0	13·0	12·25	27·0	25·4
B033*o*	356	224	40·8	64·7	44·3	70·5	16·5	26·2
B034*c*	186	195	63	60·0	13·5	12·8	24·5	23·3
H013*o*	340	—	44·8	—	40·5	—	13·9	—
H015*c*	210	—	58·5	—	19·8	—	22·3	—

o = obese; *c* = control.

TABLE 2. METHODS OF ESTIMATION OF ADIPOSITY OF OPERATED RATS

rat no.	weight minus weight of control	weight minus 'ideal weight'	fat content of operated minus control
126	79	82	83·5
130	198	226	213
165	89	80	77
173	4	41	58
176	76	68	85
230	153	171	154
266	82	56	57
267	270	239	230
B033	170	132	133

twice to three times that of the control animal. This represented in most cases a maximum level of intake, which was maintained, often with little alteration, for several weeks and then gradually declined, until eventually little difference could be detected between the intake of the obese and control animals. When the food intake returned to normal levels, the degree of obesity was apparently maximal, and any growth observed merely reflected the normal increase shown by the control animal. These points are illustrated in figure 4. The hyperphagic period in animal 184 lasted about 60 days, although it was temporarily interrupted at 30 days. The temporary

fall in intake was probably due to slight change in environmental conditions to which these animals are very sensitive. It coincided with a rather less marked reduction of intake by the control animal. Although a few of our very obese animals never returned to a level of food intake as low as that of their controls, we repeatedly observed that increases of the order of 50% over control weight were maintained

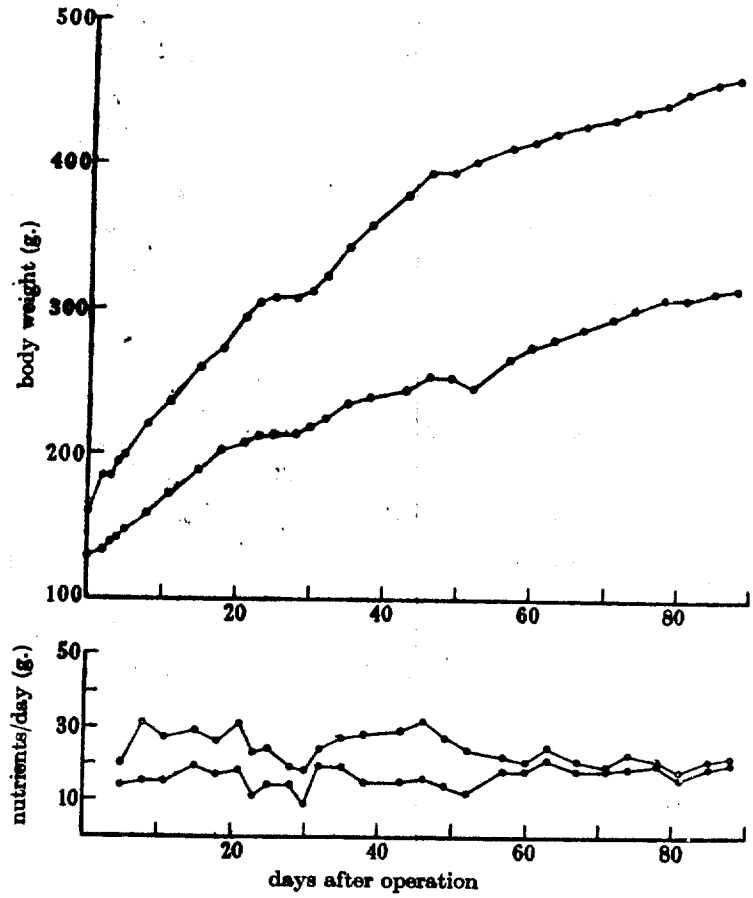

FIGURE 4. Growth and food intake of operated rat 184 and control rat 183 for 90 days from operation. o 184; • 183.

apparently indefinitely on a hardly detectable excess of food intake. At the same time it was clear that this represented an equilibrium dependent upon the presence of excessive fat depots, and not a permanent recovery from the hyperphagic disturbance, as any reduction of weight by restricted diet resulted in an immediate return of the hyperphagia when unrestricted feeding was allowed.

Reactions to adulterated diets

In a preliminary experiment we tested Adolph's findings, keeping as exactly as possible to his criteria for animal weight and period of test. Four groups each of five young male albino rats between 150 and 300 g. were given in turn either powdered

stock diet, or the same diet diluted with 25, 50 or 75 % of kaolin by weight. The test periods were each of 7 days, food intake being measured only during the last 4 days, and a week was allowed on normal diet between each test. The allocation of groups to diets was in accordance with a Latin square. Figure 5 shows the mean daily intake of all four groups expressed as total bulk eaten, and as calculated nutrient intake, on the assumption that intestinal absorption is unimpaired. (Separate tests have shown that even after 4 weeks on a 75 % kaolin diet, the rat does not show any significant failure of absorption.)

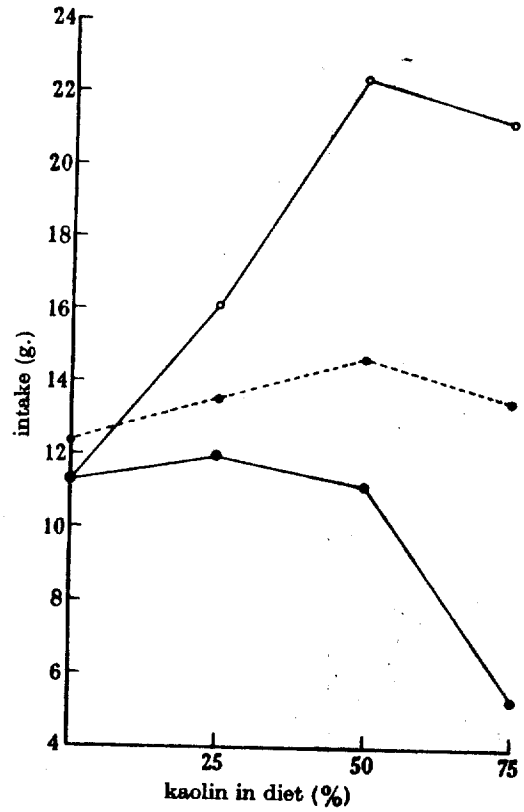

FIGURE 5. Effect of dilution of diet with kaolin on daily intake of normal male rats. o—o Total solids eaten; •—• nutrient intake; • - - • water intake.

Analysis of variance showed no significant change in nutrient intake to 50 % adulteration, but a highly significant fall in intake at 75 % of kaolin, thus confirming Adolph's finding, at least on young animals.

The difference in reaction between obese and normal animals to adulterated diets was most typically shown with 50 % kaolin mixtures, and when the obesity was fully developed. In figure 6 it will be seen that the control animal (183) restored its nutrient intake within about a week, and maintained a steady weight little different from its former level. About 4 weeks was necessary before the nutrient intake of the obese animal equalled that of the control. At the end of this time the weight

became steady, but the obesity was considerably decreased. Further reduction of weight by restricting the diluted diet was followed on derestricting by restoration of this steady weight level, which appeared to be characteristic for the individual and the particular diet.

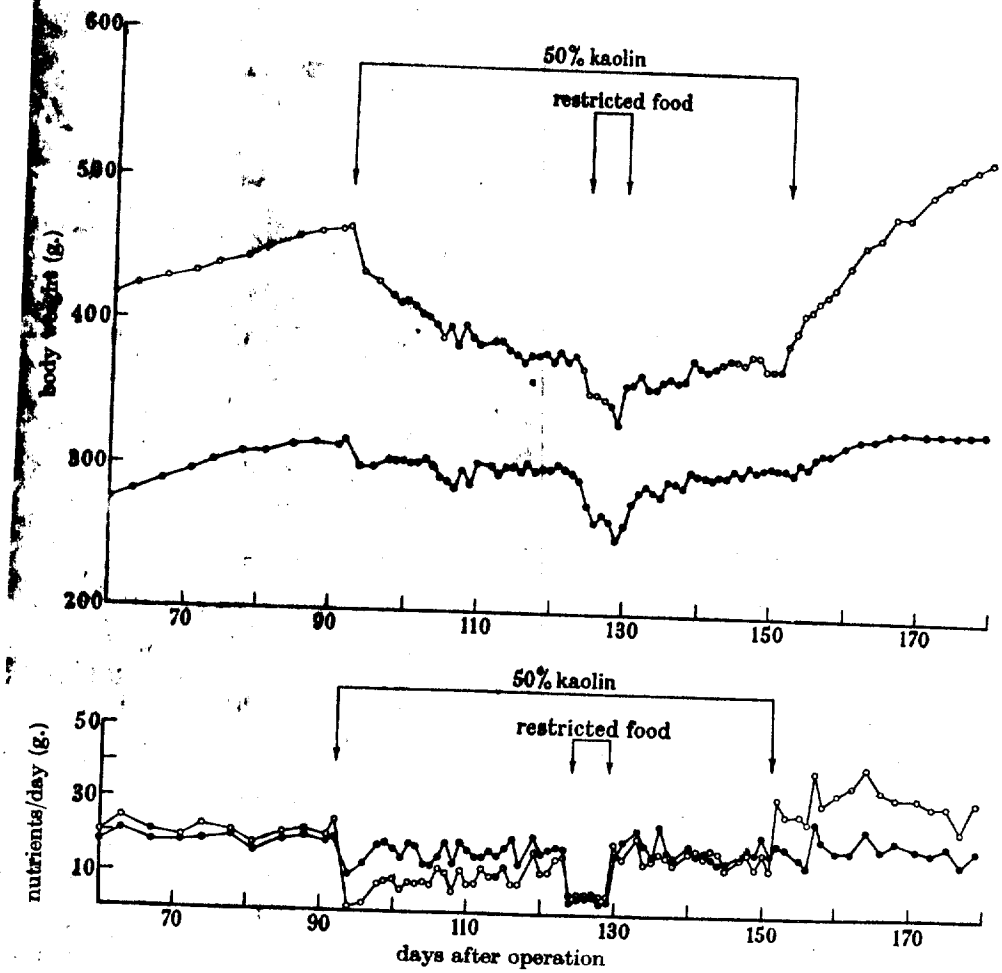

FIGURE 6. Reaction of animals 184 (operated) and 183 (control) to 50% kaolin mixture. o 184; • 183.

If the kaolin diet was given during the initial hyperphagia, before the obesity was fully developed, the reaction differed markedly. The adjustment of intake was much more rapid, and little fat appeared to be lost. In figure 7, animal 207 lost about 50 g. in apparent body weight on the first 2 days, during which it ate no food. As it had until then been forcing 40 g. of food a day into its distended gut, this probably merely reflected gut emptying. Little more weight was lost, and the food intake, as in the previous case, remained identical with the control until unrestricted feeding of undiluted food was restored, when the hyperphagia reappeared in its original intensity.

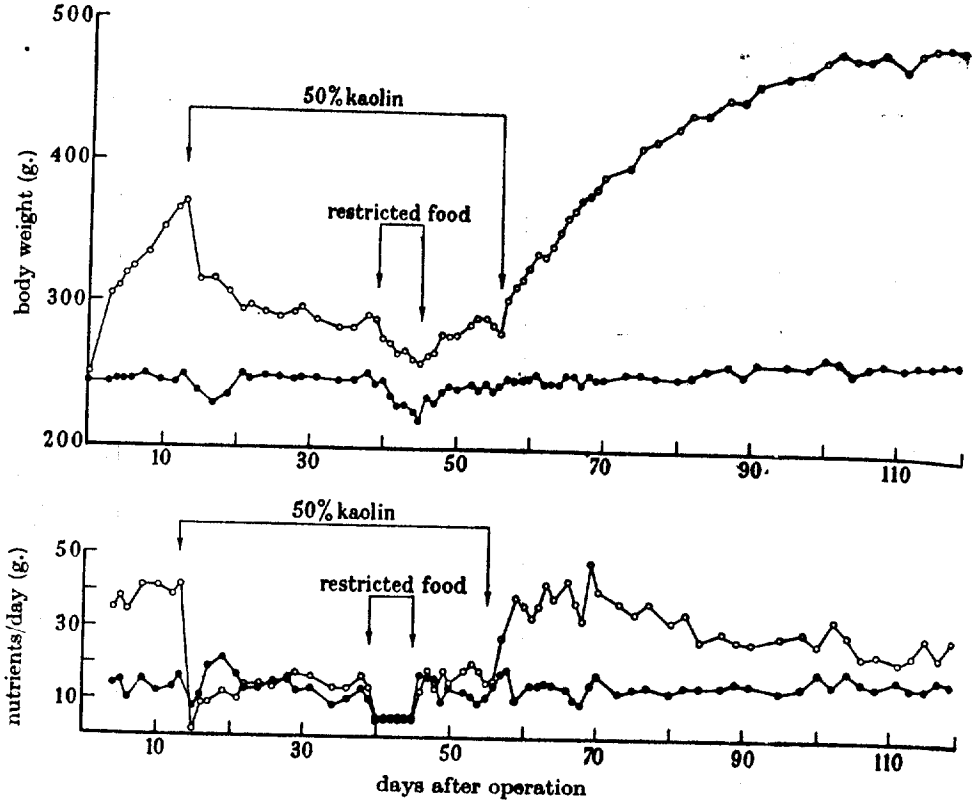

FIGURE 7. Reaction of animals 267 (operated) and 268 (control) to 50% kaolin diet introduced during hyperphagia. o 267; • 268.

Reactions of unoperated animals to adulterated diets

The observation that the older, rather fatter animals tended to react to test diets in a manner more like the operated rats than like the young ones originally studied, suggested a reinvestigation of the behaviour of 'naturally' fat rats. 102 male rats of weights varying from 150 to 450 g. were given the 50% kaolin mixture for 14 days, by which time they all appeared to have reached a steady weight. In figure 8 the weight lost on the kaolin mixture is plotted against the initial weight, of which it is clearly a function. It will be seen that animals below 300 g. can, on the whole, maintain their weight on 50% kaolin; when the diet was diluted to 75% kaolin, only young growing rats below 200 g. could tolerate this without weight loss.

Adolph, who used young rats exclusively, found that deprivation of food either compulsorily or voluntarily as a result of refusal of adulterated diets, was not followed by increased intake on return to unrestricted feeding. He concluded that 'previous privation of food is little or no stimulus to eating'. Again, our experience has been different with young and old animals. Figure 9 shows the mean daily food intake of two groups of five male rats during the last of their 4 weeks on 75% kaolin, and after their return to normal stock diet. The rats of initial weight of the

order of 200 g. showed little increase in intake during recovery, but the older rats showed clear hyperphagia. Analysis of variance shows the difference 'between weeks' in the heavier animals to be significant. In this experiment the younger animals had lost little or no weight during the kaolin feeding. Moreover, when the

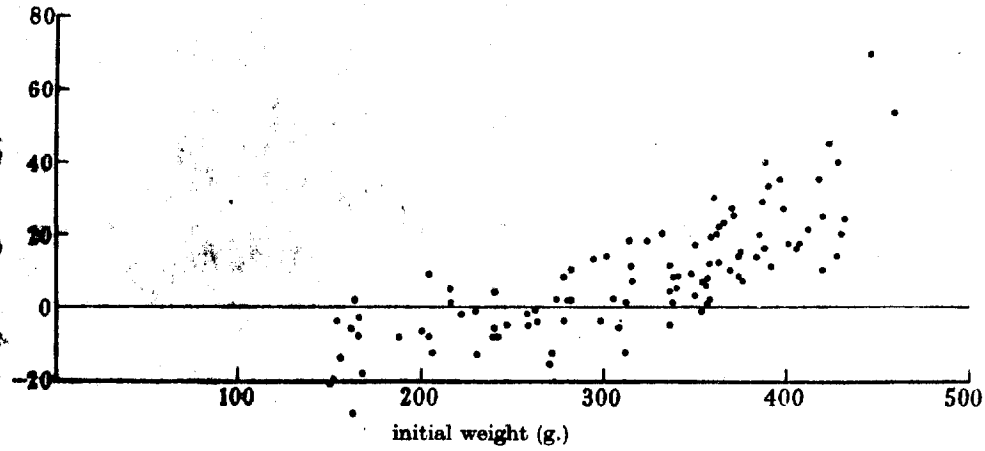

FIGURE 8. Weight loss of unoperated male rats fed for 14 days on 50 % kaolin diet. Animals which continued to grow are indicated by a negative weight loss.

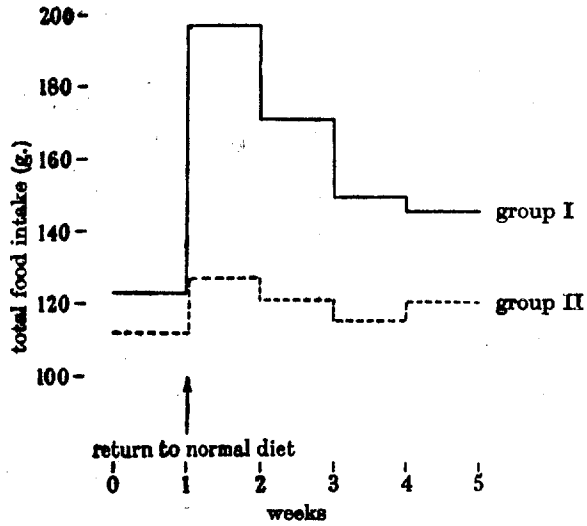

FIGURE 9. Mean weekly food intake of groups of 5 unoperated male rats on return to stock diet, after 4 weeks feeding on a 75 % kaolin mixture. Group I, mean initial body weight 372 g. Group II, mean initial body weight 227 g.

deprivation of food was compulsory, there was still no sign of hyperphagia in the young animals on return to unrestricted feeding. In figure 10 the daily food intake of three groups of five animals of mean weight approximately 400, 350 and 200 g. are compared with similar control groups recorded at the same time. The experimental groups were restricted to a daily intake of 5 g. of food for 3 days, then 10 g.

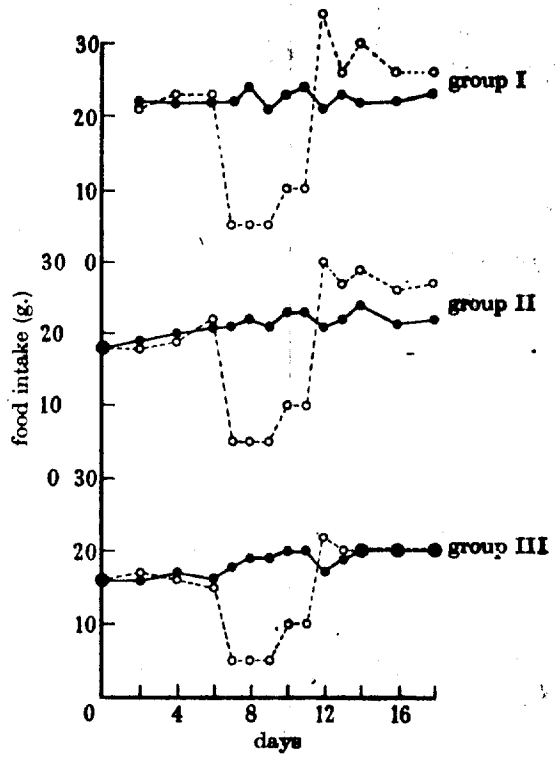

FIGURE 10. Increased food intake in normal rats on unrestricted feeding following partial starvation. Groups of five males with control groups of similar weight. Group I, initial body weight 382 to 421 g. Group II, initial body weight 332 to 361 g. Group III, initial body weight 184 to 207 g.

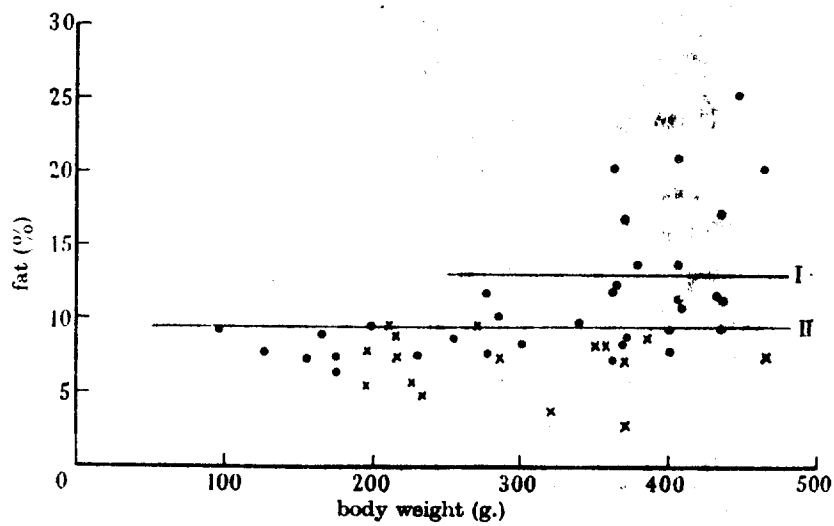

FIGURE 11. Reduction in percentage body fat of unoperated rats maintaining a steady weight on adulterated diets. • Control rats on stock diet. × Rats fed on the 75% kaolin mixture. The final fat percentage is plotted against the initial weight on stock diet, for comparison with the controls. I, II, maximum recorded fat percentage in animals eating 50% and 75% kaolin diets.

for a further 2 days. On return to unrestricted feeding, the heavier groups showed hyperphagia, the lightest group none.

It was therefore decided to investigate the normal fat content of our colony rats at different weights, and figure 11 shows the result of thirty-four fat analyses, plotted against body weight. The range of percentage fat found increases markedly with increasing weight and age, although as the precise age of the animals was unknown an exact statement of the relationship with age is impossible. The stock was young and growing, however, and in general, weight was a function of age. On the same diagram the percentage body fat found in animals at the end of the two previous experiments with kaolin diets is plotted against the weight at the beginning of the experiment. It will be seen that there is a marked reduction in the range of body fats recorded in rats above 300 g. fed on 50 % kaolin diet, while all the rats fed on 75 % kaolin mixture lost fat down to the level characteristic of 200 g. animals.

Discussion

Brobeck's work made it clear that the obesity in his rats was due to over-eating, which decreased in many cases as the fat stores increased, only to reappear after starvation. However, he drew no conclusions as to the physiological mechanism involved. As he says (1946): 'Expressed as a problem in thermodynamics, the situation appears to be fairly clear—the more the animal eats, the more obese it becomes. But, expressed in physiological terms as a problem in the regulation of energy exchange, the situation is still obscure. The question, 'Why does the animal with hypothalamic lesions eat so much extra food?' immediately raises the questions, 'Why does the normal animal eat food? What determines how much it will eat? What changes in the internal environment set the animal to eating, and what changes are associated with satiety?' A partial answer to these questions seems to be offered by a study of the differences in reaction shown by thin and fat animals in our feeding experiments. First, is hyperphagia really the essential feature of the disturbance caused by hypothalamic lesions, as is suggested by Brobeck, and by the many descriptions of 'enhanced appetite', 'voracious, tigerish appetite', 'high degree of voracity', and so on, in the literature? Such behaviour does occur post-operatively, or after starvation, but in fully developed obesity the excess food intake is never striking. Certainly the animals show no sign of voracity. In our experiments the degree, and even the nature of the reaction to a change of diet, was conditioned by qualities in the food which were almost without effect in a young, normal animal. Far from being voraciously omnivorous, the fat animals appeared to be more than usually discriminative, the discrimination increasing with the obesity. Thus, the overall effect of the hypothalamic lesion seemed to be to emphasize two components of the urge to eat, a primitive voracious one, and a more discriminative component, either of which might be dominant according to the state of the fat stores. The possibility that the rejection of the kaolin diets by the fat animals was due to the gut being distended by the diluent is slight, as some refused the diets altogether for several days, and few ate enough at first to produce even normal distension. On the other hand, they were capable of an intake of

a more attractive diet amounting to two or three times normal. The rejection appeared to depend upon smell, and taste, and to be a conditioned response of the type usually described as appetite.

In marked contrast was the behaviour of the young rats. Adolph, who concentrated on these, concluded: 'Food acceptance and the urge to eat in rats are found to have relatively little to do with a local condition of the gastro-intestinal canal, little to do with the organs of taste, and very much to do with quantitative deficiencies of currently metabolized materials.' We confirmed that a chemical control of food acceptance did appear to be dominant in the 200 g. rat. Refusal of food on the presentation of unpalatable diets very quickly resulted in a degree of hunger sufficient to compel the acceptance of the diet. Excessive intake, which would have resulted in obesity, could not be induced. The emergence of the characteristic responses of the hyperphagic animal was seen in a proportion of the older unoperated rats studied, apparently the 'naturally' fat ones. It seems reasonable to assume that the superficially simpler feeding reactions of the young rat are the result of closer co-ordination of the different aspects of food acceptance rather than a real difference in mechanism.

The apparent differences in behaviour may be explained on the following hypothesis. The hyperphagia exhibited by rats with hypothalamic lesions appears to represent a primitive unconditioned urge to eat, which may be described as hunger. Normally this is inhibited by a hypothalamic satiety mechanism, which is directly sensitive to changes in the blood brought about by the ingestion of food. This hypothalamic mechanism appears to be particularly effective in the young rat, but becomes less well co-ordinated with increasing age, or after operative damage, and the failure in control of blood metabolites is then reflected in wider variability in fat depots. In such rats the palatability of food has an effect on the weight considerably greater than is seen in the young animal, and becomes the principal determinant of the degrees of obesity on unrestricted feeding. The feeding reactions of naturally fat animals are so similar to those after hypothalamic operation as to suggest that the difference is only one of degree, and that modification of the hypothalamic control of energy intake may be a prerequisite of obesity in general.

It is a pleasure to acknowledge my indebtedness to Miss H. M. Bruce, of the National Institute for Medical Research, who was responsible for much of the quantitative information recorded in this paper, and to Dr A. S. Parkes, F.R.S., for constant encouragement and advice. My thanks are due to Dr W. L. M. Perry, of the National Institute for Medical Research, for testing the statistical significance of the results.

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Fibrogenous Effect of Bentonite Clays

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The N. Madhviladze S. P. Kipiani (Tbilisi)

Institute of Labor Hygiene and Industrial Diseases

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Clays of montmorillonite composition -- bentonites -- owing to their hydrophilic nature, and their colloidal, adsorption, emulsifying, degreasing, and other properties, find wide use in various branches of industry and in agriculture (farming).

The mining of Askansk bentonites in the Georgian S.S.R. is carried out by the open-pit method. Dust formation in extracting them is not high; this is explained, evidently, by the high moisture content (32 - 36%) of the commercial mineral. At the same time, in the course of processing Askansk clay, we observed a considerable release of dust at the Makharadze bentonite powder plant. The dust concentrations exceeded, by tens, and sometimes, by hundreds, of times the maximum permissible amount. The millers and packers of the finished products work under particularly unfavorable conditions from this point of view. A study of the dispersion of the dust suspended in the air indicated that specks of dust, with the diameter of the particles reaching up to 2 microns, comprise 57.8%; those from 5 to 10 microns -- 17.4%; and those above 10 microns -- 24.8%. A relatively high percent of particles with a diameter of more than 10 microns is associated with the property of bentonites to stick together during contact of the specks of dust with one another and to form agglomerations (clusters). The content of silicon dioxide in Askansk bentonites comprises 53%; it is found to be, basically, in a bound state with oxides of various metals, for the main part, aluminum; the content of free silicon dioxide in the suspended dust of Askansk clay fluctuates from 3.8 to 9.8%.

Taking into account that only individual papers have been devoted to the

fibrogenous effect of bentonite clays (E.O. Kossovskii; N. M. Makhviladze; Sakula; Cartner and Herzog), we considered it important to make a study of this problem clinically and experimentally. A survey was made of 96 workers in the production of Askansk bentonites, including 31 packers, 6 millers, 8 operators, 23 fitters and electricians at the plant, and 28 open-pit workers that served as a control groupl the period of service on the part of 54, in the given industry, did not exceed 4 years; in 42, it fluctuated from 5 to 9 years. The period of service on the part of the millers and packers, who have to carry out their obligations under conditions of the greatest dust content in the air, comprised, on the average, 4 years. The comparatively short period of service is explained, apparently, by the fact that we were not able, in a single case of these observed, to disclose any clinical-radiological changes in the lungs. At the same time, studies were carried out on the functions of external respiration, on the albumin fractions of the blood serum, and on the content, in it, of hexoses, and neuramino acids, and also on the phagocytic activity of leucocytes in vitro. No statistically reliable difference was disclosed between the basic indices for the function of external respiration (vital capacity of the lungs, respiratory minute volume, and the maximal pulmonary ventilation)in 35 workers in dust-associated occupations, and in 25 open-pit workers. In the packers and millers, however, there was observed a reduction in the output vigor of up to 3.5 ± 0.12 liters/second, which is reliably distinguished ($P = 0.001$) from the same index in open-pit workers. Following the injection of a 0.1% solution of atropine sulfate, the output vigor in the packers and millers increased, on the average, by 0.57 liters/second. Consequently, it may be assumed that the influence of high dust concentrations of Askansk clay produces, in the workers, bronchospasm effects.

Our investigations have shown that a prolonged influence of Askansk clay dust, in addition to this, brings about a reduction ($P = 0.001$) in the level of albumins, and an increase in the content of $alpha_2$ and gamma-globulins in the blood serum. There was also ascertained, in the workers, an increase in the content of neuramino acid in the blood ($P = 0.001$), which reached 77.1 ± 8 mg % with 61.2 ± 1.4 mg % in the control group. It should be noted that in the literature exist indications pointing to the sensitivity of glucoproteins to the influence of fibrogenous dust on the human organism(M. Timar; A.A. Lomova) and on that of animals (I.A. Gel'fon and co-workers). Askansk clay dust, in its influence on the organism, brings about a reduction in the phagocytic activity of leucocytes in vitro (in the capacity of a phagocytosis object, use was made of an emulsion of a dead daily culture of Staphylococcus aureus). Thus, if, in the control group, the phagocytic number is equal to 74.97 ± 5.2, then in the packers and millers, it is reduced to 55.48 ± 1.5 ($P < 0.001$). The depressing effect of Askansk clay dust on the phagocytic activity of leucocytes has been confirmed by us in an experiment on white rats after 2 weeks, 1, 3, 6 and 9 months following the intratracheal administration of 70 mg of it (Table 1).

Table 1

Phagocytic Activity of Leucocytes in White Rats in the Intratracheal Administration of Askansk Clay Dust

Groups and Periods of Dustiness	Phagocytic Number	Phagocytic Index
Control		
Experimental		
2 weeks		
1 month		
3 months		
6 months		
9 months		

In order to study the character and degree of phagocytosis for Askansk clay dust, white mice were administered, intraperitoneally, 10 mg of it with a content of 6.4% free silicon dioxide. A parallel experiment was conducted with dust from pure crystalline quartz. As may be seen from Table 2, phagocytosis from Askansk clay dust takes place considerably more actively than in the case of quartz dust; this is attested to by the indices both of the phagocytic number, as well as of the phagocytic index. In addition to this, upon administering Askansk clay dust, the destruction of the phagocytes was established only during the first 3 periods of observation (,3, 6 and 24 hours), and in the remaining periods (48 and 72 hours), it is absent in contrast with the quartz dust; in the administration of the latter, the destruction of the cells is observed with a growing intensity during all the periods of the experiment. The vacuolization and absorption of the residues of the destroyed cells, which we had noted during the administration of the quartz dust, are completely absent in the administration of Askansk clay dust. Thus, Askansk clay dust is characterized by a considerably less pronounced biological aggressiveness than is quartz dust.

Table 2

Phagocytosis Indices for Askansk Clay and Quartz Dusts (M $\pm$ m)

Type of Dust	Period of Exudate Extraction (in hours)	Phagocytic Number				Phagocytic Index			
		neutrophyl	monocyte	macrophage	Overall	neutrophyl	monocyte	macrophage	overall
Askansk clay									
Quartz									

In a histological investigation of the lungs of white rats after a month following the administration, into the trachea, of Askansk clay dust, in an amount of 70-100 mg, there were disclosed a weakly pronounced catarrhal

bronchitis, symptoms of peribronchitis, in places hyperplasia of the peribronchial follicles, a slight hyperplasia of the lymphoid tissue around the blood vessels, and the formation of fine foci of dust cells. There was disclosed a large amount of miliary, submiliary, and larger confluent dust-cell foci consistint of a mass of coniophages containing the finest dust particles. There was noted a proliferation of argyrophilic fibers in the dust-cell foci, and alveolar partitions, particularly around the bronchi and blood vessels. In large foci, in places, the argyrophilic fibers penetrated inside the dust-cell foci in the form of individual, rather thick strands, and in the finer ones, they formed a dense network. Around the dust-cell foci were found numerous, very fine, collagenic fibers, part of which penetrated into them. A small increase in the amount of collagenic fibers was observed around the large and medium bronchi and blood vessels. A focal thickening of the alveolar partitions close to the dust-cell foci took place as a result of an increase in the amount of collagenic fibers in them.

After 3 months, there was recorded a slight amplification of the catarrhal bronchitis and a hyperplasia of the peribronchial and perivascular lymphoid tissue. There was an increase in the amount of dust-cell foci in which were encountered individual giant cells. In larger foci, the proliferation of the collagenic fibers intensified. Fine collagenic fibers, which were disclosed in the alveolar partitions, around the blood vessels and bronchi, were arranged in a fine, circular layer. In places, along with the thickening of the alveolar partitions, emphysema was noted.

After 6 months, symptoms of catarrhal bronchitis somewhat increased. There was a continuation of the proliferation and thickening of argyrophilic fibers both in the foci, as well as around the bronchi, blood vessels, and in the alveolar partitions. There grew a number of fine, collagenic fibers around the dust-cell foci,and inside them; there was an increase in the

collagenic fibers in the thickened alveolar partitions, and there took place a proliferation of a few collagenic fibers around the fine and very fine dust foci. A thickening of the alveolar partitions took place as a result not only of the focal proliferation, in them, of fine collagenic fibers, but also, because of the multiplication, in them, of histiocytes; among the latter, there was a certain increase in the number of polynuclears. Nevertheless, there was determined a small intensification of focal emphysema.

After 9 months, there was a still greater proliferation, and there increased in number the thickened argyrophilic fibers, which clearly limit the dust-cell foci of varying sizes. Along the periphery of the latter, there was an increase in the amount of collagenic fibers. There was an increase, to a certain extent, in peribronchial and perivascular sclerosis. In many dust-cell foci there continued to be a multiplication of histiocytic elements. In the peribronchial follicles there was an increase in the amount of miliary, submiliary, in places confluent, foci of cells of an epithelioid character. As before, in the alveolar partitions the proliferative process was moderately pronounced.

In this manner, pathomorphological investigations indicated that the intratracheal administration of Askansk bentonite clay dust in white mice brings about a development of a moderate pneumochlerosis, and the formation of dust-cell foci, with symptoms of a weakly pronounced sclerosis in them.

Conclusions

1. The influence, over a course of a number of years, of high concentrations of Askansk clay dust, may bring about in the organism of workers that come in contact with it, a number of changes: bronchospasms, disproteinemia, an increase in the content of neuramino acid in the blood serum, and a reduction in the content of the phagocytic activity of leucocytes.

2. The intratracheal administration of Askansk clay dust brings about, in white rats, a development, in the lungs, of a moderate, diffuse penumosclerosis, with the formation of dust-cell foci.

3. Askansk clay dust, in comparison with crystalline quartz dust, actively phagocytizes, and its destroying effect on coniophages is weakly pronounced.

4. The results of the investigations make it possible to acknowledge the production of Askansk bentonite clays as being potentially conioso-hazardous. It must be placed among the number of industries in which the workers are subject to a preliminary and periodic medical examination once in 2 years.

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К ВОПРОСУ О ФИБРОГЕННОМ ДЕЙСТВИИ БЕНТОНИТОВЫХ ГЛИН

С. П. Кипиани (Тбилиси)

Институт гигиены труда и профзаболеваний им. Н. Махвиладзе

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Глины монтмориллонитового состава — бентониты — благодаря гидрофильности, коллоидным, адсорбционным, эмульгирующим, обезжиривающим и другим свойствам широко применяются в различных отраслях промышленности и сельского хозяйства.

Разработка асканских бентонитов в Грузинской ССР производится карьерным способом. Образование пыли при добыче их невелико, что объясняется, очевидно, высокой влажностью (32—36%) полезного ископаемого. В то же время в про-

цессе переработки асканской глины нами наблюдалось значительное выделение пыли на заводе бентопорошков г. Махарадзе. Концентрации пыли в десятки, а иногда сотни раз превышали предельно допустимую величину. В особо неблагоприятных условиях с этой точки зрения работают мельники и упаковщики готовой продукции. Изучение дисперсности взвешенной в воздухе пыли показало, что пылинки с диаметром частиц до 2 мк составляют 57,8%, от 5 до 10 мк — 17,4%, свыше 10 мк — 24,8%. Относительно высокий процент частиц диаметром более 10 мк связан со свойством бентонитов слипаться при соприкосновении пылинок друг с другом и образовывать конгломераты. Содержание двуокиси кремния в асканских бентонитах составляет 53%; она в основном находится в связанном состоянии с окислами различных металлов, главным образом алюминия; содержание свободной двуокиси кремния во взвешенной пыли асканской глины колеблется от 3,8 до 9,8%.

Учитывая, что фиброгенному действию бентонитовых глин посвящены лишь единичные работы (Е. О. Коссовский; Н. М. Махвиладзе; Sakula; Cartner и Herzog), мы сочли важным изучить этот вопрос в клинике и эксперименте. Обследовано 96 рабочих производства асканских бентонитов, в том числе 31 упаковщик, 6 мельников, 8 операторов, 23 слесаря и электрики завода и 28 рабочих карьера, служивших контрольной группой, стаж работы на данном производстве у 54 не превышал 4 лет, у 42 колебался от 5 до 9 лет. Стаж работы мельников и упаковщиков, которым приходится выполнять свои обязанности в условиях наибольшей запыленности воздуха, составлял в среднем 4 года. Сравнительно небольшим стажем объясняется, по-видимому, то, что нам ни в одном случае у обследованных не удалось обнаружить каких-либо клинико-рентгенологических изменений в легких. Одновременно были изучены функции внешнего дыхания, белковые фракции сыворотки крови и содержание в ней гексоз и нейраминовой кислоты, а также фагоцитарная активность лейкоцитов in vitro. Статистически достоверной разницы между основными показателями функции внешнего дыхания (ЖЕЛ, МОД, МВЛ) у 35 рабочих пыльных профессий и 25 рабочих карьера не выявлено. Однако у упаковщиков и мельников отмечено снижение мощности выхода до 3,5±0,12 л/сек, достоверно отличающееся (P=0,001) от того же показателя у рабочих карьера. После инъекции 0,1% раствора сернокислого атропина мощность выдоха у упаковщиков и мельников увеличилась в среднем на 0,57 л/сек. Таким образом, можно полагать, что воздействие высоких концентраций пыли асканской глины вызывает у работающих явления бронхоспазма.

Наши исследования показали, что длительное воздействие пыли асканской глины, кроме того, вызывает снижение (P=0,001) уровня альбуминов и повышение содержания α_2- и γ-глобулинов в сыворотке крови. У рабочих было констатировано также повышение содержания нейраминовой кислоты в крови (P=0,001), достигающего 77,1±8 мг% при 61,2±1,4 мг% у контрольной группы. Следует отметить, что в литературе имеются указания на чувствительность глюкопротеидов к воздействию фиброгенной пыли на организм человека (М. Тимар; А. А. Ломова) и животных (И. А. Гельфон с соавторами). Пыль асканской глины при воздействии на организм вызывает понижение фагоцитарной активности лейкоцитов in vitro (в качестве объекта фагоцитоза брали эмульсию убитой суточной культуры золотистого стафилококка). Так, если у контрольной группы фагоцитарное число равно 74,97±5,2, то у упаковщиков и мельников оно снижено до 55,48±1,5 (P<0,001). Угнетающее действие пыли асканской глины на фагоцитарную активность лейкоцитов подтверждено нами в эксперименте на белых крысах через 2 недели, 1, 3, 6 и 9 месяцев после интратрахеального введения 70 мг ее (табл. 1).

Для изучения характера и степени фагоцитоза пыли асканской глины белым мышам внутрибрюшинно вводили 10 мг ее с содержанием 6,4% свободной двуокиси кремния. Параллельно был поставлен опыт с пылью чистого кристаллического кварца. Как

Таблица 1

Фагоцитарная активность лейкоцитов у белых крыс при интратрахеальном введении пыли асканской глины

Группы и сроки запыления	Фагоцитарное число			Фагоцитарный индекс		
	$M \pm m$	t	P	$M \pm m$	t	P
Контрольная	46,6±0,5			3,4±0,4		
Опытные:						
2 недели	71,5±3,6	3,0	0,001	5,3±0,4	3,0	0,001
1 месяц	24,0±1,6	3,0	0,001	1,9±0,33	2,9	0,01<P<0,02
3 месяца	37,0±3,4	2,8	0,02	2,1±0,36	2,4	0,02<P<0,05
6 месяцев	37,0±1,7	3,0	0,001	1,7±0,22	3,1	0,001
9 »	34,3±1,5	3,0	0,001	1,7±0,3	3,0	0,001

Таблица 2

Показатели фагоцитоза пыли асканской глины и кварца ($M \pm m$)

Вид пыли	Срок извлечения экссудата (в часах)	Фагоцитарное число				Фагоцитарный индекс			
		нейтрофил	моноцит	макрофаг	общее	нейтрофил	моноцит	макрофаг	общее
Асканская глина	3	12,0±2,2	9,0±2,1	8,0±2,5	29,0±1,9	1,5±0,17	1,9±0,5	2,3±0,3	9,7±0,4
	6	13,0±2,6	5,8±1,2	8,6±1,5	27,4±1,8	2,8±0,2	4,2±0,5	4,2±0,4	3,5±0,15
	24	14,0±1,9	8,0±1,2	16,0±1,2	38,0±4,3	3,9±0,5	3,6±0,8	7,4±1,1	5,5±0,5
	48	11,0±0,5	8,0±0,9	15,0±4,1	34,0±5,4	4,2±0,76	4,3±0,18	4,1±0,53	4,2±0,48
	72	14,0±2,5	11,0±3,6	12,0±1,1	37,0±4,5	4,4±0,77	6,8±0,8	7,5±1,3	6,6±0,8
Кварц	3	1,3±0,8	2,9±1,0	1,8±1,0	6,0±2,3	1,0±0,0	1,0±0,0	1,0±0,0	1,0±0,0
	6	1,6±1,5	4,4±0,0	2,9±0,9	2,0±0,0	1,3±0,25	3,1±1,1	3,1±1,4	2,1±0,47
	24	3,3±2,6	8,3±2,5	12,4±1,9	24,0±4,1	2,4±0,7	3,5±1,2	3,8±1,2	3,2±1,7
	48	3,0±1,1	10,0±0,5	21,0±3,3	34,0±3,6	2,7±0,5	3,5±0,31	3,6±0,27	3,3±0,36
	72	3,0±0,55	8,0±2,4	14,0±1,6	25,0±3,1	4,0±0,48	4,7±0,5	4,1±0,43	4,3±0,15

видно из табл. 2, фагоцитоз пыли асканской глины протекает значительно активнее, чем пыли кварца, о чем свидетельствуют показатели как фагоцитарного числа, так и фагоцитарного индекса. Кроме того, при введении пыли асканской глины разрушение фагоцитов установлено лишь в первые 3 срока наблюдения (3, 6 и 24 часа), а в остальные сроки (48 и 72 часа) оно отсутствует в отличие от пыли кварца, при введении которой разрушение клеток наблюдается с нарастающей интенсивностью во все сроки эксперимента. Вакуолизация и поглощение остатков разрушенных клеток, отмеченные нами при введении пыли кварца, совершенно отсутствуют при введении пыли асканской глины. Таким образом, пыль асканской глины характеризуется значительно менее выраженной биологической агрессивностью, чем пыль кварца.

При гистологическом исследовании легких белых крыс через месяц после введения в трахею пыли асканской глины в количестве 70—100 мг обнаружены слабо выраженный катаральный бронхит, явления перибронхита, местами гиперплазия перибронхиальных фолликулов, небольшая гиперплазия лимфоидной ткани вокруг кровеносных сосудов и образование мелких очажков из пылевых клеток. Выявлено большое количество милиарных, субмилиарных и более крупных сливающихся клеточно-пылевых очажков, состоящих из массы кончофагов, содержащих мельчайшие пылевые частицы. Отмечено разрастание аргирофильных волокон в клеточно-пылевых очажках и альвеолярных перегородках, особенно вокруг бронхов и кровеносных сосудов. В крупных очажках местами аргирофильные волокна прорастали в глубину клеточно-пылевых очажков в виде отдельных, довольно толстых тяжей, а в более мелких они образовывали густую сеть. Вокруг клеточно-пылевых очажков найдены многочисленные тончайшие коллагеновые волокна, часть которых прорастала в них. Небольшое увеличение количества коллагеновых волокон наблюдалось вокруг крупных и средних бронхов и кровеносных сосудов. Очаговое утолщение альвеолярных перегородок близ клеточно-пылевых очажков происходило за счет увеличения количества в них коллагеновых волокон.

Через 3 месяца зарегистрировано небольшое усиление катарального бронхита и гиперплазии перибронхиальной и периваскулярной лимфоидной ткани. Увеличилось количество клеточно-пылевых очажков, в которых встречались отдельные гигантские клетки. В более крупных очажках разрастание коллагеновых волокон усиливалось. Тонкие коллагеновые волокна, обнаруживавшиеся в альвеолярных перегородках, вокруг кровеносных сосудов и бронхов располагались тонким циркулярным слоем. Местами наряду с утолщением альвеолярных перегородок отмечалась эмфизема.

Через 6 месяцев явления катарального бронхита несколько нарастали. Продолжалось разрастание и утолщение аргирофильных волокон как в очажках, так и вокруг бронхов, кровеносных сосудов и в альвеолярных перегородках. Нарастало количество тонких коллагеновых волокон вокруг клеточно-пылевых очажков и в них самих; больше стало коллагеновых волокон в утолщенных альвеолярных перегородках, происходило разрастание немногочисленных коллагеновых волокон вокруг мелких и мельчайших пылевых очажков. Утолщение альвеолярных перегородок происходило за счет не только очагового разрастания в них тонких коллагеновых волокон, но и размножения в них гистиоцитов, среди которых несколько увеличилось количество полинуклеаров. Вместе с тем определено небольшое усиление очаговой эмфиземы.

Через 9 месяцев еще больше разрослись и увеличились в своем числе утолщенные аргирофильные волокна, четко ограничивающие клеточно-пылевые очажки различной величины. По периферии последних увеличивалось количество коллагеновых волокон. Несколько нарастал перибронхиальный и периваскулярный склероз. Во многих клеточно-пылевых очажках продолжалось размножение гистиоцитарных элементов. В перибронхиальных фолликулах возросло количество милиарных, субмилиарных, местами сливающихся очажков из клеток эпителиоидного характера. По-прежнему в альвеолярных перегородках пролиферативный процесс был умеренно выражен.

Таким образом, патоморфологические исследования показали, что интратрахеальное введение белым крысам пыли асканских бентонитовых глин вызывает развитие умеренного пневмосклероза и формирование клеточно-пылевых очажков с явлениями слабо выраженного склероза в них.

Выводы

1. Воздействие в течение ряда лет высоких концентраций пыли асканской глины может вызвать в организме рабочих, контактирующих с ней, ряд изменений: бронхоспазм, диспротеинемию, повышение содержания нейраминовой кислоты в сыворотке крови, понижение содержания фагоцитарной активности лейкоцитов.

2. Интратрахеальное введение пыли асканской глины вызывает у белых крыс развитие в легких умеренного диффузного пневмосклероза с образованием клеточно-пылевых очажков.

3. Пыль асканской глины по сравнению с пылью кристаллического кварца активно фагоцитируется, ее разрушающее действие на кониофаги выражено слабо.

4. Результаты исследований позволяют признать производство асканских бентонитовых глин потенциально кониозоопасными. Оно должно быть отнесено к числу производств, рабочие которых подлежат предварительному и периодическому медицинскому осмотру, раз в 2 года.

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THE ADSORPTION OF VITAMIN A BY SODIUM BENTONITE[1, 2]

By D. H. Laughland and W. E. J. Phillips

Abstract

A study has been made of the reaction whereby a blue adsorption complex is formed when an organic solvent solution of vitamin A is added to sodium bentonite. In addition to a physical adsorption of the vitamin on sodium bentonite a chemical alteration occurs in the vitamin A molecule which results in the formation of anhydro vitamin A. The adsorbed material may be removed from the sodium bentonite by extraction with diethyl ether but the blue color is dissipated and a secondary reaction product is formed. This latter compound exhibits the characteristics of triene conjugation and exhibits maximum absorption at about 290 mμ. Equations have been developed for the spectrophotometric analysis of the binary mixtures encountered in the reaction and satisfactory agreement has been obtained between calculated and observed values.

Introduction

It was recognized many years ago by Takahashi and Kawakami (5) that conjugated polyene pigments were adsorbed by acid earths and that a blue reaction product was formed. Zechmeister and Sandoval (8) demonstrated that in the case of phytofluene the reaction was irreversible since the material eluted from the column did not possess the original absorption curve. The blue adsorption product which resulted from the treatment of vitamin A with an acid clay was eluted with alcohol by Meunier and Vinet (3). It was suggested that this substance, which had an absorption maximum at 330 mμ, was a diether formed by the elimination of one mole of water from two moles of vitamin A. Weil-Malherbe and Weiss (7) studied the colored reaction products formed between a number of alumino-silicate materials and various compounds possessing a conjugated double bond structure. It was concluded that color formation with vitamin A results from the ability of a conjugated system to donate an electron to the acid clay and form an easily hydrolyzed salt.

Experimental

Methods

In general, the procedure which was followed in this work was to add a Skellysolve B (Skelly Oil Co., Kansas City, Mo.) solution of vitamin A to a suitable amount of sodium bentonite and to let the mixture stand in the dark. Excess vitamin A was removed by extracting the sodium bentonite with Skellysolve B. Diethyl ether was found to be an effective solvent for the elution of the product from the adsorbent. The blue color of the adsorption complex was immediately dissipated upon the addition of ether to the bentonite and the product was obtained as an orange colored oil.

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[2] *A preliminary report on this work was given at the annual meeting of the Chemical Institute of Canada at Toronto in June 1954.*

Measurements of light absorption were made in a Model DU Beckman spectrophotometer by employing the hydrogen discharge tube and silica cells. The vitamin A concentrate used in most of this work was a synthetic vitamin A acetate preparation of Distillation Products, Rochester, N.Y. and contained 1.5×10^6 I.U. vitamin A per gram. The concentrate was saponified prior to its use in most of these experiments in order to remove irrelevant material.

Formation of Adsorption Complex

An indication of the rapidity with which vitamin A is removed from solution is given in Table I. Forty milliliters of Skellysolve B solution of vitamin A alcohol containing 21.22 μmoles (μ M.) of the vitamin was added to various amounts of sodium bentonite. Each flask was shaken 100 times by hand and the suspension was centrifuged. The amount of vitamin A remaining in the supernatant was determined and the amount adsorbed was calculated by difference.

TABLE I

ADSORPTION OF VITAMIN A ALCOHOL BY SODIUM BENTONITE

Wt. of bentonite (gm.)	μM. of vitamin A alcohol			% of total adsorbed
	Added	Recovered	Adsorbed	
0.0	21.22	21.22	0.00	0.00
0.2	21.22	17.72	3.50	16.49
0.4	21.22	12.49	8.73	41.14
0.6	21.22	8.86	12.36	58.24
0.8	21.22	6.44	14.78	69.65
1.0	21.22	3.72	17.50	82.47

In all cases in which vitamin A was present, the sodium bentonite was colored a deep blue. It may be observed from these results that under the conditions specified above, most of the vitamin A present in solution was adsorbed by the sodium bentonite.

Skellysolve B solutions of vitamin A acetate, alcohol, and palmitate were prepared to contain approximately the same number of μM. per ml. Aliquots of these solutions were added to 0.5 gm. samples of sodium bentonite and each flask was shaken 100 times by hand. The results in Table II indicate that the

TABLE II

ADSORPTION OF VITAMIN A ALCOHOL, ACETATE, AND PALMITATE ON SODIUM BENTONITE

Vit. A alcohol (μM.)		Vit. A acetate (μM.)		Vit. A palmitate (μM.)	
Added	Ads./gm.	Added	Ads./gm.	Added	Ads./gm.
3.66	5.36	3.52	2.74	3.64	2.44
7.32	11.66	7.04	3.52	7.28	2.46
10.98	16.08	10.56	5.08	10.92	2.84
14.64	21.56	14.08	4.96	—	—
18.30	26.48	17.60	5.80	18.20	3.80

adsorption affinity of sodium bentonite is greater for vitamin A alcohol than for either vitamin A acetate or palmitate. Later work indicated that the shaking procedure employed in this experiment was not sufficient to permit the attainment of equilibrium because the amount of solute adsorbed per gram adsorbent did not reach a constant value.

The length of time required for equilibrium to be reached was determined by shaking samples of vitamin A acetate, palmitate, and alcohol with sodium bentonite for various periods of time. The results of an experiment in which aliquots of solutions of vitamin A alcohol, vitamin A acetate, and vitamin A palmitate were shaken with 0.5 gm. samples of sodium bentonite in a volume of 40.0 ml. Skellysolve B are shown in Fig. 1. The millimolar concentration of vitamin in the Skellysolve B solutions was 1.05 for the alcohol, 0.533 for the acetate, and 0.621 for the palmitate. The samples were shaken on a mechanical shaker and the amount of vitamin A adsorbed by the sodium bentonite was calculated by difference. These results indicated that the amount of vitamin adsorbed per gram of sodium bentonite reached a maximum at about 90 min. The data also demonstrated that the adsorption affinity of sodium bentonite is much greater for vitamin A alcohol than for the acetate or the palmitate. Similar data were obtained for β-carotene and it was found that the same time relationship prevailed although only about 5.0 μM. of β-carotene was adsorbed per gram of sodium bentonite under equilibrium conditions.

An attempt was made to assess the adsorption affinity of sodium bentonite for vitamin A alcohol by the application of the Freundlich and Langmuir equations to the experimental data. At low concentrations of the vitamin it appeared that the requirements for these equations were met in a satisfactory manner. The amount of the vitamin adsorbed per gram of sodium bentonite was calculated by difference from the amount remaining in the supernatant

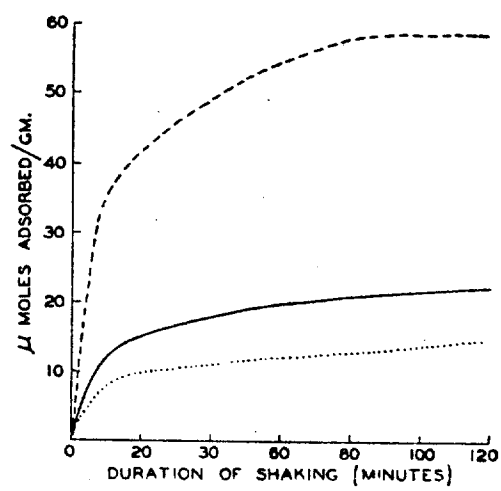

FIG. 1. Adsorption of vitamin A alcohol, acetate, and palmitate on sodium bentonite.

solution at equilibrium. It was observed, however, that results were not reproducible at high concentrations of the vitamin and it became obvious that the vitamin was undergoing a chemical change during the adsorption process in addition to being physically adsorbed on the bentonite.

Mechanism of the Reaction

The blue adsorption complex which is formed when a Skellysolve B solution of vitamin A alcohol is added to sodium bentonite can be removed with ethanol but much more readily by means of diethyl ether. When vitamin A is added at a concentration which is only slightly in excess of that required to saturate the adsorbent, then only unchanged vitamin A is found in the Skellysolve B fraction. If, however, the concentration of vitamin A is such that a large excess is present then the absorption maximum due to the vitamin is shifted to a higher wave length and additional maxima are evident.

The Skellysolve B fraction, under these conditions, is composed of a mixture of vitamin A and anhydro vitamin A. Anhydro vitamin A, which is formed by the dehydration of vitamin A alcohol according to Shantz, Cawley, and Embree (4) possesses the absorption curve shown in Fig. 2. Curves are also shown for vitamin A alcohol and for a mixture composed of equal weights of the alcohol and anhydro forms of the vitamin. In a mixture of this type the absorption ratio A 370 mμ/A 350 mμ is related inversely to the amount of vitamin A present in the mixture and may be used, in view of the negligible contribution of the alcohol form to the absorbancy at 370 mμ, as a reliable estimate of the relative proportion of the two forms present in a mixture.

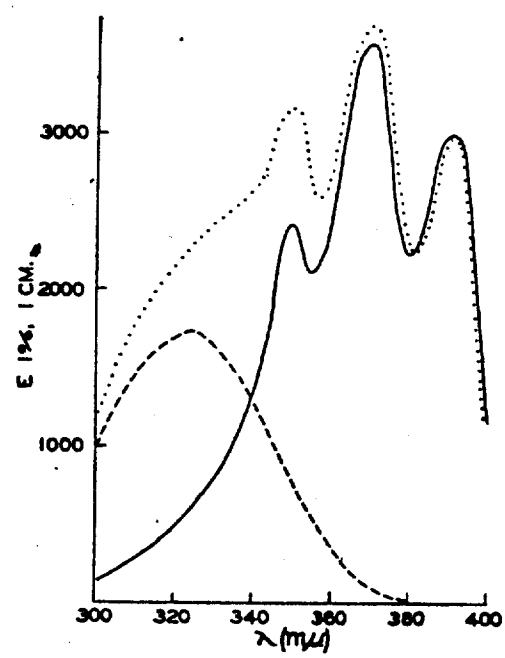

FIG. 2. Absorption curve of vitamin A alcohol and anhydro form.

A number of experiments were carried out which differed with regard to the ratio of vitamin A to sodium bentonite and to the length of time the preparation was allowed to stand before removal of the supernatant fraction. It was not possible to reproduce the results of these experiments in a satisfactory manner until the nature of the mixture was realized and suitable equations applied for the estimation of the two forms of the vitamin. These considerations are illustrated by an experiment in which 71.5 mgm. of vitamin A alcohol, dissolved in 60.0 ml. of Skellysolve B, was added to 3.0 gm. of sodium bentonite. The sample was shaken on a mechanical shaker and 5.0 ml. aliquots were removed at various periods of time. The aliquot was centrifuged and the absorption curve of the supernatant fraction was determined in ether solution. These data are shown in Fig. 3.

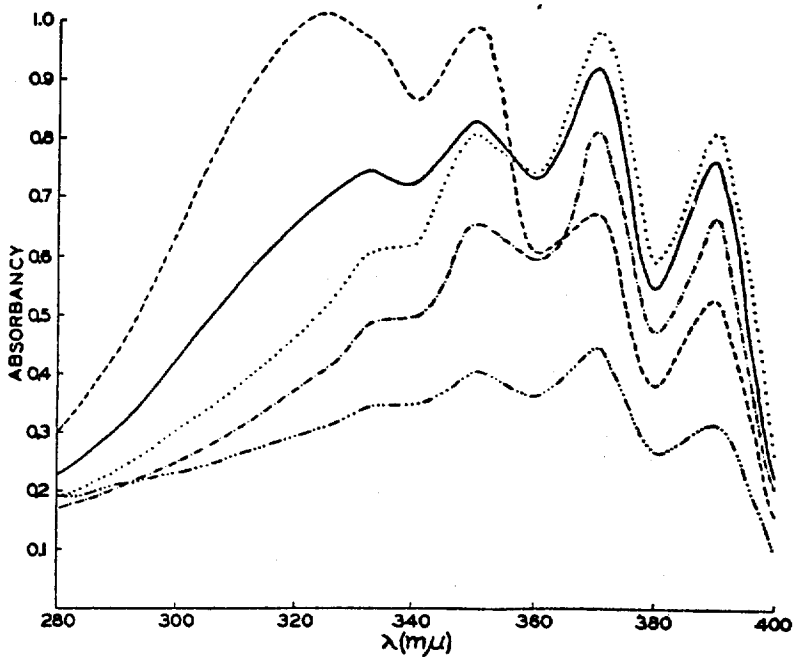

FIG. 3. Skellysolve B extracts of sodium bentonite treated with vitamin A.

The results indicate that the amount of vitamin A in the supernatant solution decreased with time and that the anhydro form was produced rapidly at first and then more slowly. This is evident from the decrease in the absorption due to vitamin A at 325 mμ and that attributed to the anhydro form at 370 mμ. It appeared that the curves were the resultant of a two component system which consisted of vitamin A alcohol and anhydro vitamin A. It is possible, using the absorption curves in Fig. 2 together with a value for the extinction coefficient of 3650 at 370 mμ for anhydro vitamin A and 1750 at 325 mμ for vitamin A alcohol, to develop equations which permit the estimation of the amount of each component in a mixture. The specific

absorption coefficient is the same for the anhydro form and for vitamin A alcohol at 339 mμ and is equivalent to a value of 1260. The second wave length selected for the calculation was 325 mμ, the absorption maximum of vitamin A alcohol. The ratio A 325 mμ/A 339 mμ was calculated to be 1.390 for vitamin A alcohol and 0.508 for anhydro vitamin A.

Let absorbancy at 339 mμ be x for the anhydro form and
y for vitamin A alcohol,

then $x + y = A$ 339 mμ

$0.508x + 1.390y = A$ 325 mμ.

These equations may be solved to give:

$x = 1.577\ A$ 339 mμ $- 1.132\ A$ 325 mμ

and $y = 1.132\ A$ 325 mμ $- 0.577\ A$ 339 mμ.

Using the value E (1%; 1 cm.) 339 mμ = 1260 for both the anhydro form and vitamin A alcohol, these equations may be modified to give:

μgm./ml. (anhydro form) $= 12.51\ A$ 339 mμ $- 9.0\ A$ 325 mμ

μgm./ml. (vit. A alcohol) $= 9.0\ A$ 325 mμ $- 4.58\ A$ 339 mμ.

Results

It is possible by the use of these equations to calculate the amount of vitamin A alcohol and anhydro vitamin A present in solution at any time after initiation of the reaction. The amount of anhydro vitamin A present in solution approximates the total amount of this component present in the reaction mixture because it has been demonstrated that anhydro vitamin A is physically adsorbed on sodium bentonite to only a slight extent under the conditions of these experiments. Although some absorption attributable to the anhydro form is found in samples of adsorbent removed from the reaction mixture prior to 10 min. after the initiation of the reaction, there is little evidence for the presence of the anhydro form after this time. The equations were applied to the data given in Fig. 3 and these results are shown in Table III. The amount of vitamin A which has been adsorbed either in the alcohol form or in the form of degradation products may be estimated by subtracting the weight of material in solution from that which was initially present.

TABLE III

THE FORMATION OF ANHYDRO VITAMIN A FROM VITAMIN A ALCOHOL

Time (min.)	Amount present		Combined wt. of two forms (mgm.)	Wt. of material adsorbed (mgm.)
	Anhydro (mgm.)	Alcohol (mgm.)		
0	0.00	71.50	71.50	0.00
1	5.46	15.50	20.95	50.54
5	8.46	8.83	17.29	54.21
15	9.36	5.34	14.70	56.80
30	7.56	4.32	11.88	59.62
90	5.16	3.72	8.88	62.62
120	4.67	3.60	8.27	63.23

In order to test the validity of the procedure which has been described, the absorption curves were constructed for each of the mixtures given in Table III and reasonable agreement was found between the calculated and observed curves. The results of such a calculation for the 30 min. sample are shown in Fig. 4.

The results indicated that the initial phase of the reaction was rapid and about 70% of the vitamin A alcohol was either physically adsorbed or converted to the anhydro within the first minute. The concentration of anhydro form in the supernatant fraction increased for about 15 min. and then decreased in amount whereas the vitamin A alcohol decreased with time throughout the entire two hour period. At the end of two hours it was calculated that about 88% of the vitamin A alcohol had been removed from solution.

It was pointed out previously that the adsorption of vitamin A alcohol on sodium bentonite resulted in the formation of a blue adsorption complex. The adsorbed material was not removed by Skellysolve B but could be readily extracted by means of diethyl ether with a dissipation of the blue color. Upon evaporation of solvent an orange colored oil was obtained which absorbed

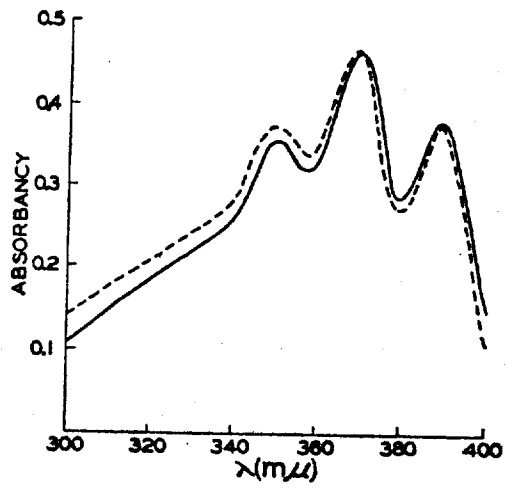

FIG. 4. Observed and calculated curves for a mixture of the alcohol and anhydro forms of vitamin A.

light most strongly in the region 200–300 mμ. Several samples of this material were prepared under a variety of conditions with respect to the ratio of vitamin A concentration to the weight of adsorbent and, although the absorption curves were dissimilar in some respects, there was an indication of a maximum at about 250 mμ and a more intense broad band at about 285–290 mμ in each sample. It was concluded after further examination of these curves that they represented a mixture of vitamin A alcohol and a degradation product of the vitamin. This unidentified degradation compound has been referred to as "product" in the text. The product could be precipitated from diethyl ether solution by the addition of ethanol and the absorption curve for

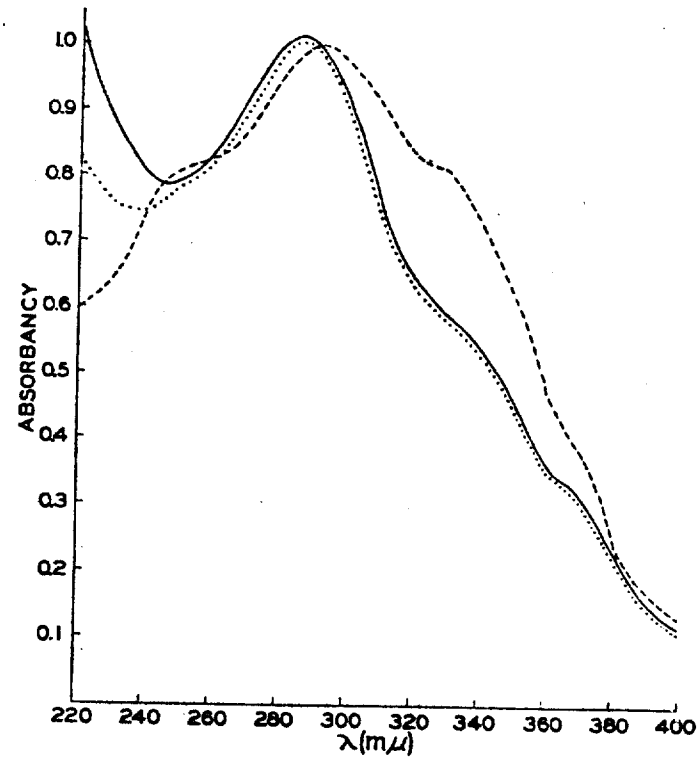

FIG. 5. Product extracted from bentonite.

this orange product is given in Fig. 5. Preliminary studies have indicated a melting point of 131° with decomposition. The extinction coefficient was calculated to be 376 at 290 mμ and 311 at 260 mμ.

During the course of the removal of solvent under vacuum from a crude ether extract of sodium bentonite product it was found that the sample crystallized spontaneously. The absorption curve of this material resembled that of subvitamin A which has been described by Embree and Shantz (1). Subvitamin A appears to have a lower extinction coefficient than the product described in this paper. Moreover, the intensity of absorption below 250 mμ is greater for subvitamin A than it is for the product. Oxygen was bubbled through an ethanol solution of this material maintained at 70° C. for 30 min. and the effect of this treatment is shown in Fig. 5. Treatment with oxygen under these conditions did not materially influence the shape of the absorption curve except at wave lengths below 250 mμ. It was observed that under conditions of destruction by light the product changed from orange through yellow to a cream color and that it became insoluble in diethyl ether.

Equations were developed to test the hypothesis that the curves for the ether extract represented a mixture of product and vitamin A alcohol. It may be observed from the curve for the product that the absorbancy at 260 mμ was equivalent to that at 325 mμ. Employing a value for the ratio A

260 mμ/*A* 325 mμ of 1.000 for the product and 0.156 for vitamin A alcohol in ether solution, equations were developed for the analysis of the binary mixture. These equations were:

$$\mu\text{gm. vit. A alcohol/ml.} = 6.77\,(A\ 325\ \text{m}\mu - A\ 260\ \text{m}\mu)$$

$$\mu\text{gm. product/ml.} = 38.17\,A\ 260\ \text{m}\mu - 6.01\,A\ 325\ \text{m}\mu\,.$$

These equations were applied to the absorption data for the ether extracts of product which were obtained using seven different types of bentonite. Twenty-five milliliters of Skellysolve B containing 15.5 mgm. of vitamin A alcohol was added to 300 mgm. of bentonite and the samples were allowed to stand with frequent stirring for 30 min. The samples were centrifuged and the Skellysolve B supernatant solution was removed. The residue was treated twice with 25.0 ml. diethyl ether and allowed to stand 30 min. before centrifuging. It was possible to demonstrate reasonable agreement between the observed curve for product and the reconstructed curve which was obtained for a mixture of product and vitamin A. The composition of the mixture was predicted from the equations given above. It would appear, therefore, that the absorption curve given in Fig. 5 closely approximates that of the unstable product.

The results of this work are shown in Table IV where the calculated values are given for vitamin A alcohol and anhydro vitamin A in the Skellysolve B solution and vitamin A alcohol and product in the ether extract. The difference between weight of material recovered in these three components and that of the vitamin A alcohol originally added represents the amount of material still adsorbed on the bentonite. It should be pointed out that the bentonite was not exhaustively extracted with diethyl ether and more of the adsorbed material would be extracted under more rigorous conditions. It was observed that the blue color of the adsorbent was most intense in samples 4 and 6 in which the concentration of anhydro vitamin A was greatest. The

TABLE IV

RECOVERY OF VITAMIN A ALCOHOL, ANHYDRO VITAMIN A, AND PRODUCT FROM BENTONITES

Sample	Skelly solution		Ether solution		Total recovered (mgm.)	Total adsorbed* (mgm.)	% Adsorbed
	Anhydro (mgm.)	Alcohol (mgm.)	Alcohol (mgm.)	Product (mgm.)			
1	0.35	9.30	0.31	1.84	11.80	5.85	37.8
2	0.25	10.40	0.34	0.84	11.83	4.85	31.3
3	0.10	9.50	0.18	2.04	11.82	5.90	38.0
4	0.55	6.80	0.26	3.48	11.09	8.15	52.5
5	0.20	9.60	0.48	4.62	14.90	5.70	36.8
6	1.75	4.65	0.24	2.15	8.79	9.10	58.7
7	0.25	5.00	0.36	2.40	8.01	10.25	66.1

* *Difference between material added and that removed in Skelly fraction.*

results suggest that vitamin A alcohol undergoes dehydration in the presence of bentonite to yield anhydro vitamin A which forms a blue adsorption complex. The sodium bentonite used in all other experiments reported in this paper is designated as sample 7 in Table IV.

Data for the chemical analysis of six of the bentonite samples are given in Table V. Samples 4 and 6 in Table IV, in which the blue color of the adsorption complex was most intense and which yielded the greatest amount of anhydro vitamin A, appear to be low in their content of Na_2O. It is not definite from these results, however, that a distinction can be made between the so-called calcium and sodium bentonites on the basis of their relative ability to form anhydro vitamin A from vitamin A alcohol. This ability may be related more properly to the mineral rather than chemical composition of the bentonite.

TABLE V

PERCENTAGE COMPOSITION OF BENTONITE SAMPLES

Chemical constituent	Sample number					
	1	3	4	5	6	7
SiO_2	60.96	52.52	50.95	57.49	51.69	65.79
Al_2O_3	18.27	16.10	16.54	20.27	18.65	19.40
Fe_2O_3	2.83	0.51	1.36	2.92	4.00	3.15
FeO	0.14	0.26	0.26	0.19	0.23	0.42
MgO	2.96	5.69	4.65	3.18	1.64	2.40
CaO	0.10	0.47	2.26	0.23	0.29	0.94
Na_2O	1.44	0.82	0.17	1.32	0.08	2.10
K_2O	0.31	0.23	0.47	0.28	0.17	0.62
H_2O*	6.56	8.16	8.28	6.85	7.89	—
H_2O†	6.78	14.68	15.01	76.3	14.82	—
TiO_2	0.08	0.16	0.32	0.12	0.16	0.16

* *Water believed to be incorporated in crystal lattice.*
† *Water which is removed during heating to 300° C.*

Discussion

The experimental data which have been described are believed to be compatible with the following theory concerning the formation of a blue adsorption complex when a Skellysolve B solution of vitamin A alcohol is treated with sodium bentonite. Vitamin A alcohol undergoes dehydration in contact with sodium bentonite to yield anhydro vitamin A which forms the blue adsorption complex. Some of the vitamin A can, however, be physically adsorbed without being converted to the anhydro form. The unstable anhydro form undergoes a further change to yield an unidentified product which absorbs light most intensely at 290 mμ. The fact that vitamin A palmitate and acetate also give a blue adsorption complex may be attributable either to hydrolysis of the esters in the presence of sodium bentonite, or to the presence of small amounts of vitamin A alcohol which occur as a contaminant in the commercial ester preparations. Hawkins and Hunter (2) have suggested that a terminal hydroxyl group is essential for anhydro formation.

The unstable product has not been identified. It resembles the subvitamin A described by Embree and Shantz (1) but the extinction coefficient of their material was considerably lower than that provisionally calculated for the product, and subvitamin A exhibits a greater intensity of absorption below 250 mμ than the product does.

Vinet and Meunier (6) have described a compound which possessed an absorption maximum at 280 mμ with an inflection at 330 mμ and a secondary maximum below 245 mμ. This material was produced by the action of perphthalic acid on vitamin A acetate in the dark. The inflection at 330 mμ was ascribed to the original system of five conjugated double bonds in vitamin A and the maxima at 280 mμ and below 245 mμ to triene and diene conjugation respectively. It is possible that the product extracted from the bentonite samples possesses a similar type of structure.

Acid clays of the bentonite type are capable of effecting marked changes in the vitamin A molecule and the initial phase of the reaction may be employed as a useful means for the preparation of anhydro vitamin A.

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THE EFFECT OF SODIUM BENTONITE ADMINISTRATION ON VITAMIN A METABOLISM IN THE RAT[1, 2]

BY D. H. LAUGHLAND AND W. E. J. PHILLIPS

Abstract

The inclusion of sodium bentonite in the diet severely limited the ability of vitamin A deficient rats to store the vitamin in the liver. This effect was observed both with vitamin A and with the provitamin β-carotene. Moreover, the liver weight per 100 gm. body weight was found to be less in most of the groups receiving sodium bentonite than in control groups. The results may be interpreted in terms of a physical adsorption of vitamin A or β-carotene on sodium bentonite present in the small intestine. Sodium bentonite cannot be regarded as an inert ingredient of the diet of rats and its prolonged administration might be expected to have deleterious effects.

Introduction

Sodium bentonite is being used to improve the pelleting characteristics of a variety of mixed feeds. It is claimed that a harder, more attractive pellet is produced when this material is added to the feed at a level of about 2.0%. Moreover, this acid clay exerts a beneficial effect by its lubricating action on the dies of pelleting machines and prolongs the life of the machines. In view of the destructive action of acid clays on vitamin A and conjugated polyenes, the mechanism of the reaction has been studied by Laughland and Phillips (1). Sodium bentonite adsorbs vitamin A to a marked degree and can effect certain chemical changes during the adsorption process. This change results in the dehydration of vitamin A alcohol and the production of anhydro vitamin A. The work described in this paper has been conducted in an attempt to provide some insight into the effects of the administration of sodium bentonite to rats.

Experimental

Materials and Methods[4]

Albino rats were obtained from the departmental stock colony and were maintained on a diet of commercial fox cubes. The vitamin A deficient diet which was used to reduce the liver reserves of vitamin A in some experiments had the following percentage composition: sucrose 65, vitamin-free casein 18, brewers yeast 8, corn oil 5, and salt (U.S.P. XIII) 4.

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Contribution No. 262, Chemistry Division, Science Service, Canada Department of Agriculture, Ottawa.

[2] *A preliminary report on this work was given at the annual meeting of the Chemical Institute of Canada at Toronto in June 1954.*

Vitamin D was added at a level of 300 I.U. per 100 gm. diet. This diet will produce gross symptoms of vitamin A deficiency in from four to five weeks if given to rats from the time of weaning at 21 days.

Liver samples were analyzed for vitamin A by saponifying the tissue with alcoholic potassium hydroxide. Five milliliters of 5.0% alcoholic potassium hydroxide was used for each gram of tissue and the sample was heated at 70° C. in a water bath until the tissue had disintegrated. The sample was cooled, extracted with Skellysolve B, and centrifuged. Vitamin A was determined by the antimony trichloride reaction after an aliquot of the Skellysolve B extract was transferred to chloroform.

Results

Two preliminary experiments were carried out to determine the effect of the incorporation of sodium bentonite in the diet of rats. The rats were maintained on a vitamin A deficient diet for a three week period prior to the initiation of the experiments in order to reduce the amount of vitamin A in the liver. There were six groups of an equal number of male and female rats. One group was maintained on the vitamin A-deficient basal diet and the other five groups were given this diet supplemented with vitamin A. In addition, sodium bentonite was included in the diet of four of the vitamin A supplemented groups at a level of either 0.5, 1.0, 1.5, or 2.5%. The rats were fed the diets ad libitum and at the end of the experimental period were killed and the livers were removed.

In Experiment 1 each group consisted of six rats and they were maintained for three weeks on the appropriate diets. Supplementation with a vitamin A acetate concentrate was at a level of 10000 I.U. per kgm. diet. In Experiment 2 each group consisted of eight rats and they were maintained for six days on the diets. Vitamin A supplementation was at a level of 20000 I.U. per kgm. The results of these experiments are given in Table I.

TABLE I

INFLUENCE OF SODIUM BENTONITE (S.B.) ON LIVER STORAGE OF VITAMIN A

Diet	I.U. of vitamin A/liver	
	Expt. 1	Expt. 2
Basal	12	8
Basal + vit. A	181	152
" + " + 0.5% S.B.	109	61
" + " + 1.0% S.B.	47	57
" + " + 1.5% S.B.	44	28
" + " + 2.5% S.B.	15	7

It was evident that, under the conditions outlined above, sodium bentonite impaired the ability of vitamin A deficient rats to store the vitamin in the liver. It should be emphasized that the liver reserves of these rats were low at the start of the experiment and that the sodium bentonite limited the absorption of vitamin A and its storage in the liver. The degree of impairment in the utilization of vitamin A appeared to be directly related to the amount of sodium bentonite incorporated in the diet.

In view of the foregoing results it became of interest to determine the effect of sodium bentonite administration to rats which were maintained on the normal diet employed in the rat colony.

An experiment was carried out with five groups, each of 10 rats of each sex. The rats were maintained on the stock cubes for a period of four weeks after weaning and were then given a diet of ground cubes to which either 0.5, 1.0, 1.5, or 2.5% sodium bentonite was added. The animals were weighed frequently during the two week period that they were maintained on the experimental diets. At the end of this time the rats were killed and 10 livers from each group were removed for analysis. The results of this experiment, given in Table II, indicated that the administration of sodium bentonite did not significantly alter the body weight of normal rats during a period of two weeks. However in all groups receiving sodium bentonite the liver weight was less than that of controls either when expressed in terms of total liver weight or as grams liver per 100 gm. body weight.

TABLE II

EFFECT OF SODIUM BENTONITE ADMINISTRATION TO NORMAL RATS FOR A PERIOD OF TWO WEEKS

Sodium bentonite in diet (%)	Mean weight			I.U. of vit. A	
	Body (gm.)	Liver (gm.)	Liver per 100 gm. body wt.	Per liver	Per gm. liver
0.0	137 ± 5.63	6.91 ± 0.33	5.04 ± 0.07	164.4 ± 12.15	24.4 ± 1.84
0.5	134 ± 2.68	5.74† ± 0.23	4.28† ± 0.07	167.6 ± 8.80	29.3 ± 1.70
1.0	131 ± 4.00	5.62† ± 0.22	4.29† ± 0.09	144.3 ± 10.95	25.9 ± 2.72
1.5	131 ± 4.02	6.02* ± 0.18	4.64* ± 0.14	134.4 ± 6.78	21.8 ± 1.33
2.5	130 ± 3.12	5.93† ± 0.14	4.57† ± 0.12	141.3 ± 9.02	24.0 ± 1.36

* *Significantly different from control at $P = 0.05$.*
† *Significantly different from control at $P = 0.01$.*

No significant change was observed in the amount of vitamin A present in the liver.

An experiment was conducted in which a stabilized form of vitamin A was used to determine if this material behaved in a similar fashion to the vitamin A ester concentrate employed in the other experiments. The vitamin A reserves of a group of normal rats were depleted by placing the rats on a vitamin A deficient diet for a period of four weeks. The rats were then divided into seven groups, each consisting of four males and four females. A negative control group was maintained on the vitamin A deficient diet and the remaining six groups were given the basal diet supplemented with 20000 I.U. of vitamin A per kgm. The vitamin A was given in the form of stabilized "Crystalets" produced by the Pfizer Co. In addition, sodium bentonite was added to the diet of the groups receiving vitamin A at a level of 0.5, 1.0, 1.5, 2.5, or 3.5%. The rats were fed ad libitum with free access to water for a period of six days and were then killed.

The results in Table III indicated that sodium bentonite at a level of 1.5, 2.5, or 3.5% in the diet significantly limited the increase in body weight which resulted from the administration of vitamin A. It was observed, moreover, that the mean liver weight of groups receiving more than 1.0% sodium bentonite in the diet was significantly lower than that of the control group. This effect was also significant when calculated on the basis of liver weight per 100 gm. body weight for groups receiving 1.0, 1.5, and 3.5% sodium

TABLE III

EFFECT OF SODIUM BENTONITE ADMINISTRATION TO RATS PARTIALLY DEFICIENT IN VITAMIN A

Sodium bentonite in diet (%)	Mean weight			I.U. vit. A	
	Body (gm.)	Liver (gm.)	Liver wt. per 100 gm. body wt.	Per liver	Per gm. liver
0.0	140.5 ± 4.82	7.15 ± 0.36	5.08 ± 0.16	232.6 ± 12.89	33.2 ± 2.60
0.5	127.5 ± 4.00	6.85 ± 0.20	5.38 ± 0.09	172.9† ± 12.78	25.6* ± 2.82
1.0	128.5 ± 5.05	5.95 ± 0.20	4.64* ± 0.11	147.3† ± 8.27	25.0* ± 1.97
1.5	124.3* ± 3.37	5.43† ± 0.19	4.38† ± 0.15	148.5† ± 14.80	27.6 ± 3.08
2.5	126.6* ± 2.49	6.00† ± 0.13	4.75 ± 0.14	155.1† ± 16.75	26.1 ± 3.08
3.5	124.4* ± 4.29	5.64† ± 0.14	4.55* ± 0.11	143.1† ± 8.04	24.2* ± 1.56
Neg. control‡	127.0* ± 3.45	6.48 ± 0.18	5.12 ± 0.18	55.1† ±12.18	8.85† ± 2.14

* *Significantly different from group receiving no sodium bentonite at P = 0.05.*
† *Significantly different from group receiving no sodium bentonite at P = 0.01.*
‡ *Group receiving neither vitamin A nor sodium bentonite.*

bentonite. Although the negative control group, which received neither vitamin A nor sodium bentonite, was significantly lower in body weight than the group receiving vitamin A, the liver weight was not changed.

The amount of vitamin A in the liver was significantly lower in all groups receiving sodium bentonite than in the group receiving vitamin A but no sodium bentonite. In the negative control and in the groups receiving 0.5, 1.0, and 3.5% sodium bentonite the effect was significant when calculated on the basis of I.U. of vitamin A per gram of liver. It would appear, therefore, that sodium bentonite interferes with the utilization of vitamin A either for purposes of growth or for liver storage.

It became of interest, in view of the influence of sodium bentonite on the utilization of vitamin A, to determine the utilization of β-carotene under similar conditions. Seven groups of rats, each consisting of six males and six females, were maintained on the basal vitamin A deficient diet which was supplemented with crystalline β-carotene at a level of 24.0 mgm. per kgm. Sodium bentonite was added to the diet at a level of 0.5, 1.0, 1.5, 2.0, or 3.0%. In addition one group was maintained on a basal diet containing 3.0% alphacel. Alphacel is a ground cellulose preparation which provides nonnutritive bulk in the diet. It is produced by Nutritional Biochemicals Corporation of Cleveland. The rats were fed the diets for a period of 14 days and were then killed and six livers from each group were removed for analysis.

TABLE IV

EFFECT OF SODIUM BENTONITE ON THE UTILIZATION OF CRYSTALLINE β-CAROTENE BY RATS

Bentonite in diet (%)	Mean weight			I.U. of vit. A	
	Body (gm.)	Liver (gm.)	Liver wt. per 100 gm. body wt.	Per liver	Per gm. liver
0	138.9 ± 5.09	6.10 ± 0.29	4.39 ± 0.09	622.5 ± 29.10	113.0 ± 8.09
0.5	147.6 ± 7.65	7.21 ± 0.63	4.88* ± 0.19	492.3† ± 26.15	75.0* ± 11.50
1.0	144.0 ± 6.72	7.21 ± 0.53	4.96* ± 0.19	495.2* ± 43.30	79.0† ± 4.68
1.5	143.2 ± 6.60	7.01 ± 0.51	4.90* ± 0.17	385.5† ± 35.9	62.0† ± 10.20
2.0	140.2 ± 6.30	6.75 ± 0.18	4.89* ± 0.19	445.0† ± 25.4	65.4† ± 2.22
3.0	139.5 ± 6.90	6.98 ± 0.52	4.95† ± 0.14	313.3† ± 53.6	53.7† ± 10.0
3.0 (alphacel)	139.3 ± 5.65	6.93 ± 0.38	4.97† ± 0.12	539.6 ± 69.0	93.7 ± 16.75

* *Significantly different from group receiving no sodium bentonite at $P = 0.05$.*
† *Significantly different from group receiving no sodium bentonite at $P = 0.01$.*

The results in Table IV indicated that there was no signicant change in the body weight or liver weight of the sodium bentonite treated groups when compared with the control group. The liver weight per 100 gm. body weight was significantly increased in all the groups receiving more than 0.5% sodium bentonite in the diet. The reason for the increase in liver weight is not readily apparent. The amount of vitamin A, when calculated either as I.U. per liver or I.U. per gram of liver, was significantly lower in the sodium bentonite treated groups than in the control. The group maintained on 3% alphacel exhibited the same significant increase in liver weight per 100 gm. body weight that was evident in the groups receiving sodium bentonite. However, the level of vitamin A in the liver was not significantly different in the alphacel group from that of the control.

Discussion

Acid clays of the type exemplified by sodium bentonite may be expected to exert several different effects when incorporated in the diet of animals. Sodium bentonite can influence the physical state of the intestinal contents in at least two ways. The bulk of the intestinal contents is increased by the feeding of sodium bentonite, and the absorption of certain nutrients may be altered by modifying peristalsis and the rate of passage of the contents through the intestine. The ability of sodium bentonite to adsorb a considerable amount of water and thereby alter the consistency of the intestinal contents is probably related to this effect. Superimposed on these two influences is the marked adsorption affinity of sodium bentonite for vitamin A and various conjugated polyenes.

The experimental work described in this paper demonstrates that sodium bentonite effectively counteracts the increase in liver reserves of vitamin A which might be expected to result from the administration of the vitamin to partially deficient rats. This effect is evident when vitamin A is administered in the form of a vitamin A acetate concentrate, as gelatin stabilized vitamin A, or as the provitamin β-carotene.

Laughland and Phillips (1) have reviewed the literature related to the formation of colored complexes when vitamin A and conjugated polyenes are adsorbed on acid clays and have made further observations on the mechanism of the reaction. In view of the great adsorptive affinity of sodium bentonite for vitamin A and β-carotene it would appear logical to assume that the vitamin A in the diet was adsorbed on the sodium bentonite. It should be recognized, however, that the ability of sodium bentonite to adsorb water and increase the bulk of the intestinal contents may be important factors in limiting the absorption of vitamin A.

The ability of sodium bentonite to adsorb various compounds is related to the acidity of the clay. The adsorption affinity for vitamin A, for example, is decreased if the acidity of the clay is reduced. This observation suggests that an alteration in the pH of the intestinal contents might be expected to influence the adsorption of vitamin A on sodium bentonite in the small

intestine. A second factor should be borne in mind in considering that physical adsorption is the prime factor which limits the absorption of vitamin A. It has been demonstrated by Weil-Malherbe and Weiss (2) that a variety of compounds may be adsorbed by acid clays. The amount of vitamin A adsorbed on sodium bentonite in the intestinal lumen would therefore be related to the relative adsorption affinity of the clay for vitamin A and other related compounds present in the diet.

The experimental work which has been described in this paper demonstrates that sodium bentonite cannot be regarded as an inert ingredient when added to the diet of rats. It has been shown to exert an effect on liver weight and to decrease the liver reserves of vitamin A under a variety of conditions. The possibility of producing symptoms of vitamin A deficiency should be considered in the administration of sodium bentonite to rats for a prolonged period of time.

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SODIUM BENTONITE

What is it?
When is it used?
What about labelling?
How is it mixed?
Does it affect Vitamin A?

by D. H. LAUGHLAND

SODIUM BENTONITE is being used by a number of feed manufacturers to produce hard, glossy pellets which resist crumbling during transportation and storage.

The small flakes of sodium bentonite have a large surface area and it is the ability to absorb large quantities of water and swell to about 20 times its original size that makes this material valuable as a bonding agent. The cost of $30 — $35 a ton in carload lots is not excessive and means about 60¢ per ton of finished feed when a level of 2% is used. This article touches on legal aspects and mixing considerations but discusses more fully some nutritional effects which have been observed.

Provisions of the Feeding Stuffs Act do not prohibit the use of sodium bentonite as a bonding agent but it must be included on the registration application and label. Claims for other functions of sodium bentonite in feeds have not been substantiated.

There is a great demand in the United States for the production of a high quality cube for the feeding of range cattle and an American milling company has described some of the practical considerations of the use of sodium bentonite. It is often difficult to obtain sufficient quantities of hydraulic or old process meals which may be used with fine grounds grains in the production of cubes. Solvent meals may be substituted but the cubes tend to crumble because of the low oil content of the formulation — sodium bentonite has been found to be very satisfactory as a hardening agent in cubes prepared from solvent meals. Bentonite is used at a level of ten to forty pounds per ton because the use of larger amounts results in the production of cubes which are too hard for range feeding. It is recommended that the bentonite be pre-mixed with the mineral or some other component of the diet in order to obtain proper dispersion.

Nutritional evidence concerning the advisability of incorporating sodium bentonite in mixed feeds is controversial.

Workers at the South Dakota State College have shown that from 1/4 to 1/3 of a pound of bentonite may be consumed daily be pregnant ewes for a considerable period of time without any evidence of toxicity. In experiments with lambs bentonite was mixed with the protein supplement at a level of 1 part of bentonite to 2 parts of the protein supplement and no effect was observed on feed efficiency or on the weight gains. It was reported that the carcass yield of lambs receiving bentonite was about 2% greater than that of controls which received no bentonite.

The addition of sodium bentonite to a commercial broiler mash at levels up to 2% apparently has no effect on growth or feed consumption up to four weeks of age according to the results published by an American research laboratory. Other experiments were conducted to study the possible effect of sodium bentonite on the utilization of protein by chicks. Chicks were fed a purified diet containing a suboptimal level of 2.5% but there was no evidence that sodium bentonite influenced the utilization of protein.

D.H. LAUGHLAND is a graduate in chemistry from the Ontario Agricultural College. He obtained a Master of Arts and Doctor of Philosophy from University of Toronto in the Department of Biochemistry. On discharge from the navy in 1945 he joined the chemistry division, science service, Canada Department of Agriculture, Ottawa. At present he is in charge of the section concerned with vitamin research in the animal chemistry unit.

Similar experiments were carried out to study the possible interrelationship of sodium bentonite, aureomicin and vitamin B_{12} in the diet of chicks, but no relationship between them could be demonstrated. A level of 2.5% sodium bentonite in the diet had no adverse effect but a level of 4% sodium bentonite resulted in poor feed conversion. An experiment in which 2.5% sodium bentonite was included in a medium energy chick diet containing 775 calories per pound indicated that the chicks receiving bentonite grew at a slightly better rate than the controls. Feed efficiency was decreased slightly by the addition of 4% sodium bentonite to this medium energy diet but this level had no effect on a high energy diet containing 977 calories per pound insofar as bodyweight at 4 weeks of age and feed utilization were concerned.

In contrast to the foregoing work it has been reported by workers at the National Institutes for Health at Bethesda, Md. that New Hampshire chicks raised on a synthetic diet containing 3% sodium bentonite and 4000 units of vitamin A per pound developed symptoms of vitamin A deficiency. An experiment was carried out in which 5% bentonite was added to the diet an only 9 chicks of the total of 36 survived to 4 weeks of age. There was no mortality in the control group in which the birds averaged 335 grams as compared to 172 grams for the birds receiving bentonite. Deficiency symptoms could be completely prevented by the addition of larger amounts of vitamin A or beta-carotene to the diet. The authors found that the addition of 5% bentonite to a practical diet exerted no detrimental effect.

Workers at the Dominion Experimental station, Swift

Current, Sask. have included sodium bentonite in a poult starter diet at levels as high as 5% but no adverse effects were noted when the diet was fortified with a safety margin over known requirements for vitamin A. In other experiments a vitamin A deficient diet was used to which the vitamin was added either as 3% dehydrated cereal grass or as vitamin A. Poults receiving the vitamin A-deficient basal diet to which no bentonite was added grew at a slower rate than those receiving vitamin A or dehydrated cereal grass. When sodium bentonite was added to the vitamin A-deficient diet weight was still further reduced by a highly significant amount and symptoms of vitamin A deficiency occurred at about the fifth week of age. Sodium bentonite did not adversely affect the body weight of poults at six weeks of age except in the case of diets which were deficient in vitamin A.

It has been known for many years that vitamin A, beta-carotene, and certain other compounds may be held in irreversible combination with sodium bentonite. The results of an experiment which was carried out in our laboratories in Ottawa and which demonstrates this fact are given in Table 1.

TABLE 1

Adsorption of vitamin A alcohol by sodium bentonite

Wt. of bentonite (grams)	Milligrams of vit. A Added	Recovered	Adsorbed	% of Total Adsorbed
0.0	6.970	6.970	0.000	0.00
0.2	6.970	5.820	1.150	16.49
0.4	6.970	4.102	2.868	41.14
0.6	6.970	2.910	4.060	58.24
0.8	6.970	2.115	4.855	69.65
1.0	6.970	1.221	5.749	82.47

A solution of vitamin A in an organ solvent (hexane) was shaken with sodium bentonite and the amount of the vitamin which was retained by the bentonite was determined. It is apparent that sodium bentonite has a great absorption affinity for vitamin A and it can be shown experimentally that vitamin A and beta-carotene are destroyed by adsorption on the bentonite and cannot be recovered in an unchanged form. Vitamin A is converted, in part at least, to a derivative called anhydro vitamin A which has no biological value.

An experiment was conducted with yearling rats which had been maintained on a vitamin A-deficient diet for a period of three weeks.

The vitamin A-deficient diet was supplemented with vitamin A at a level of 4,500 I.U./pound and sodium bentonite was fed at levels up to 2.5% of the diet. The results in Table 2 demonstrate that as the level of sodium bentonite in the diet was increased ,the ability of the rats to absorb vitamin A from the intestine and store it in the liver was severely impaired.

TABLE 2

Influence of sodium bentonite on liver storage of vitamin A by rats

Diet	Units of vit. A/liver Expt. 1.	Expt. 2.
Basal	12	8
Basal + vit. A	181	152
Basal + vit. A + 0.5% S.B.	109	61
" " 1.0% "	47	57
" " 1.5% "	44	28
" " 2.5% "	15	7

Results similar to these have been obtained when beta-carotene was incorporated in the diet as a source of vitamin A. In addition to the inability of the rats to store vitamin A in the liver they grew at a slower rate than the controls and the liver weight of rats receiving sodium bentonite and supplementary vitamin A was less than that of the rats receiving vitamin A but no sodium bentonite. These results are shown in Table 3.

TABLE 3

Effect of sodium bentonite administration to rats partially deficient in vitamin A

Present S.B. in diet	Mean Wt. Body	(grams) Liver	Units of /liver	Vit. A /gm. liver
0.0	140.5	7.15	233	33
0.5	127.5	6.85	173	26
1.0	128.5	5.95	147	25
1.5	124.3	5.43	148	28
2.5	126.6	6.00	155	26
3.5	124.4	5.64	143	24

In similar experiments carried out with chicks a vitamin A-deficient basal diet composed of white corn, solvent extracted soybean oil meal, wheat middlings, fish meal, meat meal, yeast, a salt mixture and vitamin D_3 was used. The accumulation of vitamin A in the livers of the chicks, which occurred when the basal diet was supplemented with vitamin A or dehydrated cereal grass, was decreased by the inclusion of sodium bentonite in the diet. The addition of sodium bentonite to the diet prevented a normal increase in body weight in the groups in which dehydrated cereal grass was used as the source of vitamin A. Since this effect was not observed in the groups receiving vitamin A it may suggest that the sodium bentonite removed some factor other than beta-carotene present in the cereal grass which is of benefit to the chick.

In a second series of experiments in which a practical diet was used we have not observed any consistent limitation in growth attributable to sodium bentonite for periods up to eight weeks of age. The diet used in these experiments was composed of mixed ground cereal grains, fish meal, skim milk powder, yeast, salts, and vitamin D_3. It was found, however, that sodium bentonite prevented the proper utilization of vitamin A and beta-carotene present in the diet. Evidence concerning the influence of sodium bentonite on vitamin A utilization by poultry over long periods of time is not available.

Present status of sodium bentonite

1. Sodium bentonite is being used by a number of feed manufacturers as a suitable bonding agent for pelleted feeds. The incorporation of sodium bentonite at a level of from 1% to 2% in the feed results in the production of attractive, firm pellets which resist crumbling during transportation and storage.

2. Sodium bentonite should not be described as an inert ingredient in feeds because it reacts chemically with vitamin A, beta-carotene and possibly with other compounds of nutritive value present in the feeds.

3. Although it is possible to demonstrate a decreased utilization of vitamin A and beta-carotene under a variety of experimental conditions, there is no good evidence at present to indicate that sodium bentonite significantly limits the body weight of chicks to 8 weeks of age on a practical diet. One possible exception may arise where dehydrated cereal grass is used as the sole source of vitamin A.

4. The possibility of producing a deficiency of vitamin A or other nutrients should be recognized if the amounts of such nutrients present in the feed are only slightly in excess of requirements.

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Histopathological Institute of the University of Zurich
(Director: Prof. Dr. J. R. Rüttner)

Electron Microscopic Studies of Capillaries in Experimental Chronic Granulomas

H. J. Leu and J. R. Rüttner

Introduction

Changes in the ultrastructure of capillaries play an important role in a variety of disorders. Lesions of the basement membrane have been given special attention. They have been found under various conditions. Best known are changes of visceral and peripheral capillaries in diabetic individuals, described as widening of the basement membrane with deposition of neutral, PAS-positive mucopolysaccharides (Angervall and Saeve-Soederbergh [1]; Banson and Lacy [2]; Funk [3]; Mohnike [4]). Similar lesions have been observed in senile hemangiomas (Spier [5]), in varicose veins (Prusik, Reinis and Riedl [6]), and in cutaneous and subcutaneous regions indurated due to chronic venous stasis (Leu and Schnyder [7]). Morphological studies of a large number of biopsies from non-specific inflammatory granulomas from various sites and of various etiology have disclosed the existence of such lesions in chronic vaginitis, chronic salpingitis, chronic granulomatous dermatitis and mastitis, ulcerated capillary hemangiomas and ulcerated papillary adenomas of the tongue (Leu and Rüttner [8]). Examination of skin, subcutis and muscles of the great toe of some autopsied aged diabetic and non-diabetic patients proved the existence of such lesions in arterioles, venules and capillaries. Neither degree nor incidence of the vascular changes were related to the incidence of diabetes in the total group studied (Leu and Rüttner [8]).

In the following experiments it was intended to demonstrate the non-specific nature of the lesions and the possibility of producing similar ones in experimental animals.

Material and Method

Chronic granulomas were produced by injection of fibrogenic mineral dust into the peritoneum of mice (RÜTTNER [9]). Groups of 30 inbred male or female mice weighing approximately 20 g received one intraperitoneal injection of 0.5 ml of a 1% sterile aqueous suspension of quartz, kaolin (bolus alba) or quartz-containing dust obtained from human silicotic lungs (particle size less than 3 μ). The animals were sacrified at regular intervals of 2 to 52 weeks. The peritoneal nodules were removed at autopsy, were embedded in paraffin and were stained with hematoxylin and eosin, van Gieson and periodic acid Schiff reagent (PAS). The kaolin nodules were in addition examined by electron microscopy. This material was prepared as follows: fixation in osmiumtetroxyde (OSO 4) 1%, buffered according to Zetterqvist, rapid dehydration in acetone, embedded in durcupane ACM (araldite), sections stained in a solution of uranyl-acetate 1% and potassium-permanganate (KMNO 4) 1% aa.

Results

Histologically the development of the nodules corresponded to the findings published earlier by RÜTTNER (9).

Tissue reactions to intraperitoneal injection of mineral dust were more or less identical regardless of the type of dust used. Several developmental stages could be distinguished. A short initial acute inflammatory stage manifested itself in an exudative and cellular reaction, beginning with mobilization and degranulation of mast-cells. This was followed by an aggregation of dust particles. At the same time phagocytosis by peritoneal macrophages set in and thus dust cell nodules composed of phagocytes and infiltrated by fibroblasts were built up. The kaolin particles were ingested by the phagocytes by way of endocytosis and were deposited in phagosomes. Subsequent necrobiosis of the phagocytes was followed by re-phagocytosis: there was endocytosis of free crystals or uptake of kaolin-containing phagosomes. Thus the development of the fibrous nodule was related to necrobiosis and to repeated phagocytosis, involving numerous generations of phagocytes. The development of collagen fibers paralleled the repeated phagocytosis and the necrobiosis of phagocytes. Bundles of collagen fibers separated and surrounded the phagocytes, thus building a barrier against further infiltration. Finally a net-work was formed containing dust-particles and cell-fragments which were gradually replaced by further formation of collagen fibers. Lymphocytes, plasmocytes and mast-cells seemed to

be of little importance in the fibrous transformation. Together with phagocytes, capillaries began to penetrate into the nodules, turning them into dust cell granulomas. The latter were gradually transformed into cell-free fibro-collagenous nodules. The initially preponderating dust phagocytosis was thus gradually overcome by fibrillogenesis, but it remained present until the late stages. The speed of transformation of the dust cell nodule into a collagen nodule depended upon the fibrogenic activity of the dust type used.

By light microscopy, PAS-positive deposits in the capillary walls of the quartz dust nodules could not be identified with certainty, but could be observed in the outer layers of the kaolin nodules. Such PAS-positive deposits were sometimes found in the walls of venules and in histiocytes, fibrocytes and giant cells of the foreign body type. These findings were, however, not conspicuous. By electron microscopy, some marked alterations were observed in the capillaries of the kaolin nodules. The most impressive changes appeared during the stage of massive fibrillogenesis, i.e. during the second and the sixth month of observation. The main differences between normal and diseased capillaries are summarized in the following tabulation:

	Normal capillaries	Capillaries in kaolin nodules
Endothelial surface	internal and external surface smooth	internal surface irregular external surface smooth
Mitochondria	scarce, preferably of perinuclear localization	scarce, often swollen with fewer cristae than normal and with loose stroma
Golgi apparatus	poorly developed	poorly developed, preferably vesicular
Endoplasmatic reticulum	preferably short, granulated profiles	slightly increased in amount, some dilated profiles and enlarged perinuclear fissures
Pericytes	nearly always present	sometimes increased in amount and enlarged
Basement membrane	continuous, single-layered, closely apposed to cells, about 30 $\mu\mu$	continuous, closely apposed to cells, partly broadened, loosened, poorly delimited, split into 2 and more lamellae.

Fig. 1. Capillary wall in a dust cell granuloma. Internal endothelial surface irregular. Mitochondria with fewer cristae than normal and with loose stroma (67/920, 15: 41 800).

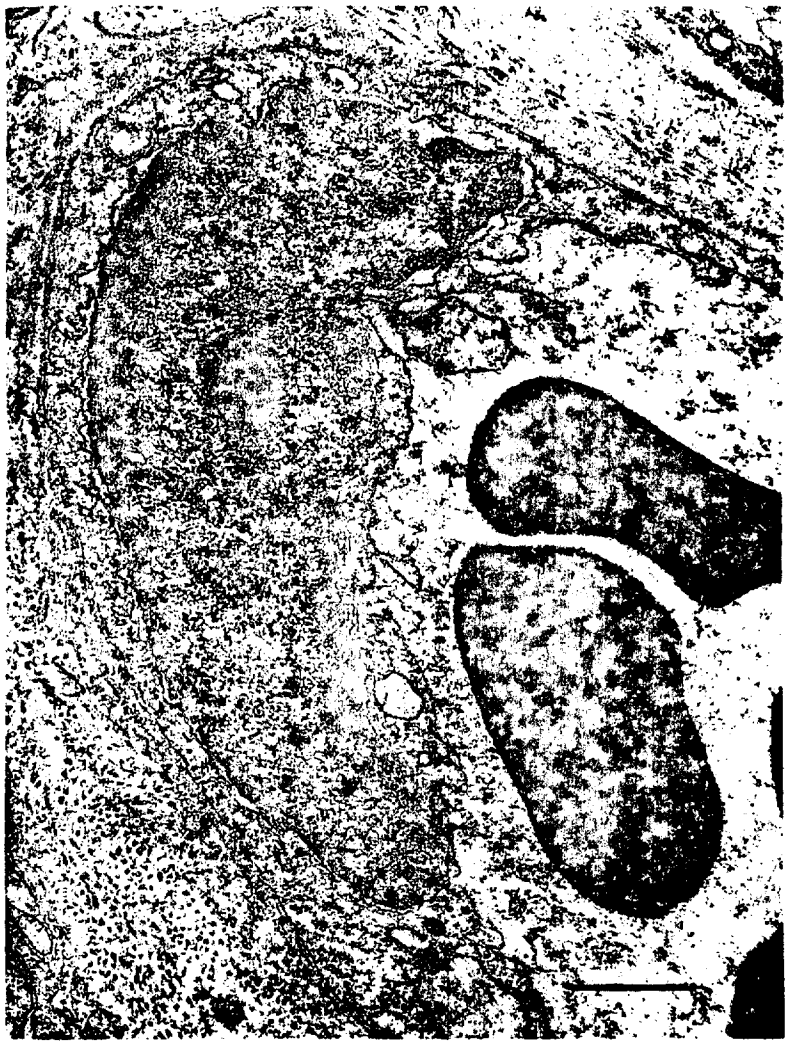

Fig. 2. Capillary wall in a dust cell granuloma. Broadened basement membrane, enlarged perinuclear fissure (67/271, 10: 16 800).

Fig. 3. Capillary wall in a dust cell granuloma. Basement membrane broadened, loosened, split into several lamellae. (67/347, 10:151 20).

Fig. 4. Capillary wall in a dust cell granuloma. Basement membrane split into several lamellae. (67/232, 13: 53 970).

Discussion

Cell-free fibro-collagenous tissue can be produced experimentally by intraperitoneal injection of fibrogenic mineral dust. The transformation of the dust cell nodules into dust cell granulomas through several developmental stages was identical for various types of dust. The fibrogenic activity of the dust type used was responsible for the speed of transformation. The main stages of the transformation were characterized by a repeated phagocytosis, necrobiosis of phagocytes and re-phagocytosis. The dust particles derived from necrobiotic phagocytes continued to possess a cytotoxic effect. Capillaries were found in the outer layers of the dust cell granulomas. They showed endothelial changes which did not occur in normal capillaries of the same animals. Kaolin (bolus alba) induced more marked lesions than quartz. The reason for this difference is unknown. The widening and splitting of the basement membrane, appearing with light microscopy as broadening and PAS-positive staining of the capillary wall, was the most impressive lesion. Although some of the vascular lesions were considerable in extent, the widening of the basement membrane could not be exactly determined because of the irregularity of the changes. The relation of the morphologic changes to capillary permeability and to the biochemistry of the basement membrane is unknown and the same may be said about the changes in the ultrastructure of the endothelial cells (irregular shape of the internal endothelial surface, changes of mitochondria, golgi apparatus and endoplasmatic reticulum.) The similarity of the lesions produced to those occurring spontaneously indicates the non-specific nature of the changes observed. Specifically their presence is not diagnostic of diabetes although they are frequently found in diabetic patients. Various factors such as hemodynamic, bacterial, toxic or metabolic influences may be responsible for the development of the lesions.

Summary

Widening and splitting of the basement membrane of capillaries was seen in dust cell granulomas produced by intraperitoneal injection of mineral dust (kaolin). These lesions thus do not constitute a specific reaction. The relation of the experimental lesions to those occurring spontaneously in a variety of conditions was discussed.

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Author's address: Dr. H. J. LEU, Hirzemätteli 12, *5400 Baden* (Switzerland).

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Inhibition of Gastric and Duodenal Proteases in the Treatment of Haemorrhage from Peptic Ulcer

I. An Experimental Study of Bentonite and other Protease Inhibitors

E. S. OLESEN
Nutrition Laboratory, Kommunehospitalet,
Copenhagen, Denmark

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The author suggests that the intermittent character of peptic ulcer bleeding depends on a sequence of clot formation and clot lysis by digestive enzymes, and that intragastric administration of adequate enzyme-inhibitors will support spontaneous haemostasis. Studies of simultaneous inhibition of pepsin and pancreatic proteases by various substances, suggest that bentonite, a clay earth mineral, has exceptional properties. It prevents lysis of standard clots by gastric juice and by duodenal aspirates in vitro, and, in animal experiments (Shay rats), has pronounced antipeptic ulcer-protective effect. A preparation of bentonite used in subsequent clinical studies by Amdrup & Olesen (1) is described.

Key-words: Bentonite; clot lysis, peptic; haemorrhage, gastroduodenal; haemostasis; pepsin inhibition; peptic ulcer bleeding; protease, pancreatic; protease inhibition; thrombolysis

E. S. Olesen, M. D., Nutrition Laboratory, Kommunehospitalet, 1399 Copenhagen, Denmark

The intermittent course of gastric or duodenal bleeding often observed, even in severe cases, indicates some spontaneous, though temporary haemostatic process. The precise mechanism of haemostasis and the factors causing relapse are not known, but a sequence of clot formation and clot lysis is a possible explanation, though not necessarily the only one.

Clot lysis may depend on the action of the powerful proteolytic enzymes, pepsin and pancreatic proteases, acting separately or sequentially; and trypsin activation of clot plasminogen may also be a significant factor. Subsequent relapse of bleeding and consequent rise in pH would inactivate pepsin, and blood trypsin inhibitors would probably reduce pancreatic proteolysis, thus facilitating clot formation and haemostasis. Eventual removal of blood from the area would allow a repetition of the cycle: a) bleeding, b) spontaneous haemostasis and temporary protection, c) clot lysis, and again a) bleeding.

Any substance which could effectively bind gastric and duodenal protease activity would tend to break this cycle by maintaining protection against proteolytic clot lysis, thus preserving haemostasis during the period of administration.

The following is an experimental study of substances potentially of clinical therapeutic value, which alone, or in combination, inhibit gastric and pancreatic proteases. Particular attention was paid to the effect of one such substance, bentonite, which was also tested for antipeptic and ulcer-protective effect in experimental animals (Shay rats).

EXPERIMENTAL

Clot-lysing activity of pepsin and pancreatic proteases, and the inhibition of this activity, were studied on fibrin and plasma clots, using a standard clot lysis technique based on that described by von Kaulla (10).

Standard clots of 0.4 ml were prepared in the narrow end of special test tubes by coagulation of either citrated human plasma or a 1 per cent solution of bovine fibrinogen. Tube ends were wetted with thrombin (Thrombin Topical®, la Roche, 10 units/ml). Calcium chloride (one-tenth volume of 0.25 M solution) was added to plasma or fibrinogen immediately before its introduction.

Solutions of enzymes and inhibitors, with appropriate buffers, a total of 6 ml in each case, were placed in the expanded open ends of the tubes. A glass bead was added, and the tubes were covered with Parafilm®.

The tubes were placed in a motor-driven rotating holder, inclined to the horizontal to secure continuous movement of the fluid. The apparatus with the tubes was placed in an incubator at 37 °C. The extent of clot lysis was read directly from a scale graduated in units of 0.01 ml, usually after 17–18 hours incubation.

In this type of experiment, varying concentrations of the inhibitor of fibrinolysis activation, epsilon-aminocaproic acid (EACA), have been incorporated in standard clots.

The fibrinolytic activity of pancreatic protease was also estimated on unheated fibrin plates (5) prepared from plasminogen containing bovine fibrinogen.

The proteolytic activity of pepsin on a soluble substrate was estimated either on haemoglobin (2) or bovine plasma (6) according to the principles described by the authors referred to. The quantity of split products soluble in trichloroacetic acid was determined spectrophotometrically from their colour reaction with Folin-Ciocalteux's reagent.

Sources of gastric and pancreatic proteases

Natural histamine-stimulated acid gastric juice, and solutions of a commercial pepsin preparation (Difco, 1 : 10,000) were used with a glycine hydrochloric acid buffer of pH 1.8.

Food-stimulated duodenal aspirates from cases with normal pancreatic function, and solutions of a pancreatic enzyme preparation (Pancreatin solubile®, Novo) were used with barbiturate buffers of pH 7.4 or 7.0.

Protease inhibitors

As pepsin inhibitors were tested the soluble anionic polyelectrolyte compounds, dextran sulphate (preparations '500' and '2000', Pharmacia, Sweden), hexametaphosphate, and a sulphated amylopectin, 'Depepsin' (G. D. Searle & Co). For inhibition of pancreatic protease activity were tested human plasma, crude eluates of defatted soya flour, fresh raw egg-white, and various combinations of these three. Since clinically, the possible destruction of pancreatic protease inhibitors by gastric digestion might be important, they were tried both in their natural state and after pre-incubation with pepsin. Combinations of inhibitors of pepsin and pancreatin were tested successively against pepsin and pancreatin.

The insoluble compound bentonite (see below) was tested as an inhibitor of both pepsin and pancreatic proteases. Further, bentonite was compared with other common adsorbents, Fuller's earth, Celite (diatomé earth), attapulgite, and glass powder. With a view to the possibility of giving bentonite with food, the influence produced by various kinds of food added to the bentonite-pepsin system was also studied.

RESULTS

Lysis of standard clots

Gastric juice and duodenal aspirates both produced lysis of fibrin clots and plasma clots, as also did solutions of pepsin and pancreatin. Hydrochloric acid alone produced no lysis of plasma clots or fibrin clots stabilized by the addition of calcium (fibrin clots without calcium were liquefied by hydrochloric acid).

Inhibition by soluble compounds

When concentrations of pepsin and pancreatin (or gastric and duodenal aspirates) were adjusted to give complete lysis of standard clots within 18 hours, the lysis could be completely prevented in both instances by appropriate concentrations of the respective soluble inhibitors mentioned previously. The fibrin plate studies, however, showed that the inhibition of pancreatic fibrinolytic activity by plasma, soya eluate, and egg-white, individually, was incomplete, but complete inhibition could be obtained by combining any two of these substances.

The incorporation of EACA in standard clots did not reduce clot lysis by gastric or pancreatic enzymes.

Simultaneous inhibition of pepsin and pancreatic protease

Pre-incubation of the natural pancreatic protease inhibitors (plasma, soya, egg-white) with pepsin at pH 1.8, followed by neutralization, produced a pepsin-dependent reduction of inhibitory capacity in all three, most marked in plasma. Addition of the soluble pepsin inhibitors, hexametaphosphate or dextran sulphate, reduced this effect, but the pepsin inhibitors themselves had their antipeptic activity reduced in the presence of plasma, egg-white, or soya eluate. Other soluble proteins, e.g. as in milk, had a similar effect.

It was, nevertheless, possible to reduce pepsin activity, and thus maintain to some extent pancreatic protease inhibition, by using relatively high concentrations of soluble pepsin inhibitors in combination with mixtures of egg-white and soya eluate.

INTERIM DISCUSSION

Though not without effect in vitro the use of mixtures of gastric and pancreatic protease inhibitors as described above, seemed not to be ideal, because of their mutual antagonistic effect. Other possible means of producing inhibition were therefore considered.

The author had previously observed characteristics similar to those of some anionic polyelectrolyte pepsin inhibitors in an insoluble mineral compound called bentonite (16). The effects of bentonite on gastric and pancreatic protease activity were, therefore, investigated.

STUDIES ON BENTONITE

Bentonite is a clay earth mineral originally found near Fort Benton in Wyoming. It is described (11) as a 'native colloidal hydrated aluminium silicate of varying composition and consisting mainly of montmorrillonite (Al_2, $4SiO_2$, H_2O), but usually containing some magnesium and iron, together with small amounts of calcium carbonate and other minerals'. It is an odourless, pale buff or cream-coloured, very fine powder of microcrystalline lamellar structure, with a very large, negatively charged surface area (20).

Enzyme binding

Bentonite, added to solutions of pepsin or natural gastric juice, and subsequently separated by centrifugation, reduced lysis of standard clots, and proteolysis of haemoglobin or bovine plasma substrates, to an extent depending on the concentrations of bentonite (Fig. 1). By means of cationic detergents, pepsin could be eluated from the bentonite sediment, with a recovery of 90–95 per cent.

The binding of pepsin to bentonite was essentially limited to pH values below 3.7, and was particularly pronounced at more acid pH (Fig. 2).

When added bentonite was not separated from the system, it reduced clot lysis similarly, but the antiproteolytic activity was much reduced in the presence of the soluble protein substrates.

At neutral pH, bentonite removed proteolytic, fibrinolytic, and clot lytic activity from duodenal aspirates and pancreatin solutions, and this effect was unaffected by prior incubation of bentonite with acid pepsin and subsequent neutralization.

The experimental results indicate that a 5

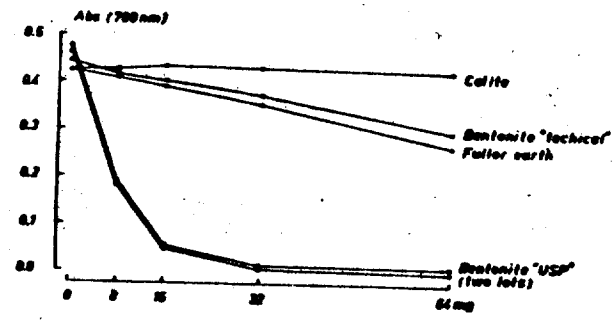

Fig. 1. Adsorption of pepsin by bentonite and other compounds. *Abscissa:* amount of adsorbing powder per ml of pepsin solution containing 1 mg per ml. *Ordinate:* pepsin activity after adsorption, measured by colour density reaction of haemoglobin split products.

per cent preparation of bentonite will reduce the peptic activity of 3–6 times its own volume of gastric juice to less than 1 per cent, and the proteolytic activity of 5 times its volume of duodenal juice to less than 5 per cent.

Various bentonites compared

A number of commercially available bentonites were examined. They showed a wide range of pepsin-binding activity. One of the most effective was 'Bentonite, USP from Fischer Laboratory Chemicals Ltd. This was chosen for the main experiments because observed pepsin-binding had been constant, and swelling properties, odour, and taste were satisfactory. It belongs to the group of so-called 'sodium-bentonites'. By contrast, a 'calcium bentonite' (Mssr. Prolabo, Paris) had poor swelling properties, less than 1/16 the pepsin-binding, and about 1/8 the pancreatin-binding capacity.

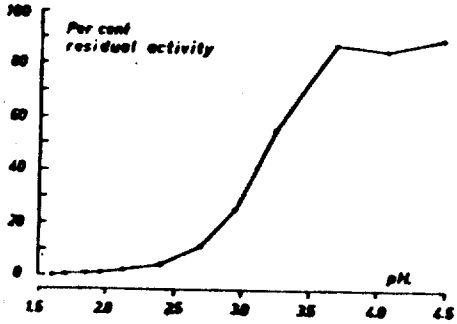

Fig. 2. Adsorption of pepsin (2.5 mg per ml) by bentonite (12.5 mg per ml) at varying pH. *Abscissa:* pH. *Ordinate:* residual activity measured at pH 1.8 on haemoglobin substrate.

Bentonite compared with other adsorbents

Fuller's earth, Celite, and glass powder were inactive, or much less active than bentonite in binding pepsin (Fig. 1).

Attapulgite, a substance similar to bentonite (19), had only one-fifth the pepsin-binding capacity, 50 mg being required to produce the same effect as 10 mg of bentonite.

Bentonite combined with food

Bentonite-pepsin binding was markedly reduced by various kinds of mechanically disintegrated, finely dispersed food, e.g. complete meals, cooked meat, boiled eggs, bread, potatoes, and other items. The effect of milk, milk dishes, raw egg-white, and blood plasma was even more pronounced. In fact, all types of protein-containing natural food reduced the binding of pepsin to bentonite. The only exception noted was heat-denatured egg-white, disintegrated and washed repeatedly with distilled water and acid buffer.

Similarly, fatty foods – heavy cream, a fat emulsion for parenteral feeding (Intralipid®), and soya bean oil emulsified in water with Tween 80® – – all reduced pepsin binding considerably. The fat-emulsifying agents, natural or synthetic, seem to be responsible for this effect of fatty foods, since soya bean oil could be emulsified directly in a bentonite suspension without affecting the binding of pepsin. Con-

sistent with this interpretation, very small concentrations of Tween 80® completely blocked the adsorption of pepsin to bentonite.

Solution of glucose, saccharose, lactose, and a malto-dextrin preparation had no effect on pepsin binding. It would thus appear that carbohydrates might be given with bentonite as a source of energy.

Sterilization of bentonite

Dry heat sterilization (140 °C for 3 hours) did not effect bentonite in any respect.

Ulcer-protective effect of bentonite in the Shay rat

Bentonite was further tested for antipeptic activity and prevention of experimental ulcer formation in Shay rats (17).

Female rats of Wistar strain were used. For 20–24 hours before operation the animals had no solid food, but were given a solution of 5 per cent glucose containing 0.4 per cent NaCl. Pyloric ligation was performed under ether anaesthesia. Post-operatively 5 ml of a 5 per cent preparation of bentonite, or in control animals 5 ml distilled water, was given by stomach tube. 12–16 hours after operation the stomachs were removed under chloroform anaesthesia after ligation of the oesophagus. Volume of gastric content, pH, free and total acidity, and pepsin activity were measured. Ulceration was rated for the groups, using a score system giving a maximum index of 400 (individual score from 0 to 4; index = average score times percentage of animals with ulcers). Results are summarized in Table I.

Bentonite had marked protective activity, despite the fact that gastric distension was slightly more pronounced, and pH was lower than in control rats. A greater incidence of gastric bleeding in control rats could explain that there was less free acidity (titration to pH 3.5) in these animals, although total acidity (titration to pH 9.2) was of the same order as in the rats given bentonite. Pepsin activity, measured on the supernatant after centrifugation of gastric contents, was markedly reduced in the bentonite-rats, being from zero to five per cent of the activity in control animals.

DISCUSSION

This was a clinically motivated experimental study of substances with inhibitory effect on gastric and duodenal proteolysis, designed to evaluate the potential of these substances in haemostatic therapy of gastroduodenal peptic ulcer haemorrhage.

Meulengracht (13, 14) touched on this idea when he noted that food intake alleviated peptic ulcer haemorrhage to some extent, but he only observed that free gastric acid in the ab-

Table I. Results of experiments with Shay rats

	Intragastric load 5 ml	No. of animals	Weight of rats (average) g	Hours after pyloric ligation	Gastric volume (average) ml	pH of gastric content*	Ulcer index for the group
I	H_2O	5	211	16	10.8	2.50	290
	bentonite 5%	5	211	16	20.4	1.34	104
II	H_2O	5	211	13	12.3	1.62	340
	bentonite 5%	5	214	13	14.3	1.52	48
III	H_2O	5	208	12	13.3	1.63	180
	bentonite 5%	4	208	12	14.9	1.58	56
IV	H_2O	5	201	13	17.8	1.45	280
	bentonite 5%	5	210	13	15.5	1.38	40
V	H_2O	5	181	13	12.9	1.62	128
	bentonite 5%	5	179	13	13.9	1.46	0

* Arithmetical mean of pH-values.

sence of food could not be a desirable state. Ashby, Anderson & Peaston (4) also saw the point in acid neutralization and reduction of peptic activity. They combined a Meulengracht diet with an intra-gastric milk drip in the treatment of peptic ulcer haemorrhage.

This present study suggested that combined treatment with soluble pepsin inhibitors and natural pancreatic protease inhibitors was likely to be relatively ineffective. The substance bentonite was found to have exceptional capacity for binding both gastric and pancreatic protease, much better than other adsorbents tested. It also inhibited pepsin as effectively as the best soluble inhibitors tested (w/w). Therefore, combinations of other pepsin inhibitors with bentonite have not been considered for clinical use at this time.

Bentonite has been used previously as a bulk laxative (12), and in the treatment of acute diarrhoea of diverse aetiology (8). A 25 per cent bentonite diet in mice is compatible with nearly normal growth (21). The development of typical benign hepatomas in mice kept over 200 days on a diet with 50 per cent bentonite, could be ascribed to choline deficiency caused by malabsorption (21). It would thus appear that bentonite can be proved in clinical trials over short periods without risk of toxic effects.

Intragastric administration of bentonite protected rats from ulcers, and the experimental findings indicate that reduction of free pepsin is the significant factor in this respect, distension and acidity being no less than in control animals. Circumstantial evidence supporting this conclusion is the laboratory finding that hydrochloric acid without pepsin had no lytic effect in the standard clots. The emphasis on pepsin, rather than acid, as the clot-lysing and ulcerogenic factor also points to a possible advantage of bentonite over antacids in reducing peptic activity. Bentonite is only a weak buffer, and probably does not interfere with the physiological acid inhibition of gastric secretion, cf. Gold & Path (9).

The acid-pepsin factor in ulcer pathology has been much studied, but the combination of acid pepsin and pancreatic protease has been relatively neglected. Arai & Necheles (3) reported that the alternate administration of acid and alkaline digestive fluids produced deleterious effects on the rat gastrointestinal tract much more pronounced than either fluid alone.

Since proteolytic tissue damage can be reinforced in this way, it is quite conceivable that blood clots could also be particularly susceptible to lysis by alternating exposure to acid pepsin and pancreatic proteases. Thus the inhibitory effect of bentonite on pancreatic protease in addition to that on pepsin could be of more than academic interest, particularly when bleeding is from a duodenal ulcer.

A further factor in the evaluation of bentonite haemostasis, not studied here, is the coagulation mechanism. Soulier (18) reports that, in vitro, small quantities of bentonite activate the contact factor (factor XII), thus accelerating coagulation. A similar compound, attaplugite, had a similar effect, and Soulier & Prou-Wartelle (19) suggested the use of attapulgite as a local haemostatic in the gastrointestinal tract. With respect to inhibition of proteolysis, bentonite, according to the present study, would seem to be the better choice.

Fibrinolysis has been linked with peptic ulcer, and the treatment of ulcer haemorrhage with antifibrinolytic agents, e.g. EACA, advocated (7, 15). In this study EACA was found to be without effect on clot lysis by the digestive enzymes in vitro.

ADDENDUM

In accordance with the experimental findings reported here, the following bentonite regimen was developed for clinical use in subsequent studies by Amdrup & Olesen (1). Bentonite USP (Fischer Laboratory Chemicals Ltd.) was selected. To guard against possible contamination by earth bacteria spores, the bentonite powder is dry heat sterilized. The finding that protein and fat foodstuff antagonized bentonite-pepsin binding, dictated that ordinary foodstuff was not simultaneously given, but some glucose is added as a source of readily available calories.

A one gallon Waring Blender (Model CB-4) is loaded with 2 kg water, 120 g bentonite, and 100 g glucose (in this order). The constituents are blended at 'medium speed' (ca. 17,000 rev/min) for 10–15 minutes. The resultant thioxotropic 'magma', if not used immediately, is refrigerator stored, but never for longer than one week.

ACKNOWLEDGEMENTS

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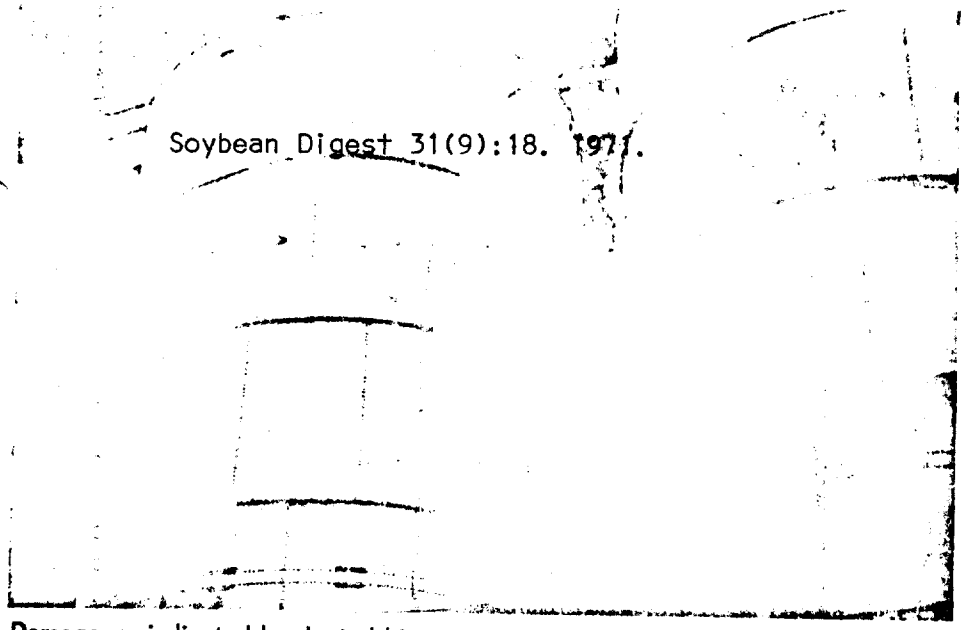


Damage, as indicated by dented bin sections, occurs when compacted meal dislodges in one slug, creating a vacuum that collapses the upper portion of the silo.

Phipps-'71
Bib No. 408

Meal flows freely at Gold Kist

By FRED V. PHIPPS JR.
Plant Manager, Gold Kist Inc.
and THOMAS HINSON
Technical Service Manager,
Clay Division, J. M. Huber Corp.

ELIMINATE 12 man-hours a day of unproductive work and all the frustrations that come when fresh soy meal compacts . . . and do this with a small capital equipment investment that pays for itself after 2 months.

That's the box score for Gold Kist Inc. in overcoming the many flow problems that are a part of producing soy meal: bridging in storage bins, fear of a "plug" blowing out the bottom of a bin, even manual digging to get the meal flowing again!

Similar compaction also had occurred during shipment, resulting in increased hopper-car-unloading time and customer production delays.

The magnitude of the problem can best be explained in terms of plant size. Gold Kist Inc. has a 2-year-old plant that has a primary soybean storage capacity of 3.5 million bushels and can process as much as 15 million bushels per year.

The fully automated plant operates around the clock, and it processes about 1,500 tons per day of beans. From these beans, oil and about 1,100 tons per day of meal are produced. It is this meal, compacting in the blending and storage bins, that has been the source of flow difficulty.

Flow of the soy meal from these containers was unpredictable, and an average of 12 manhours was consumed each day to overcome compaction and to keep the meal flowing.

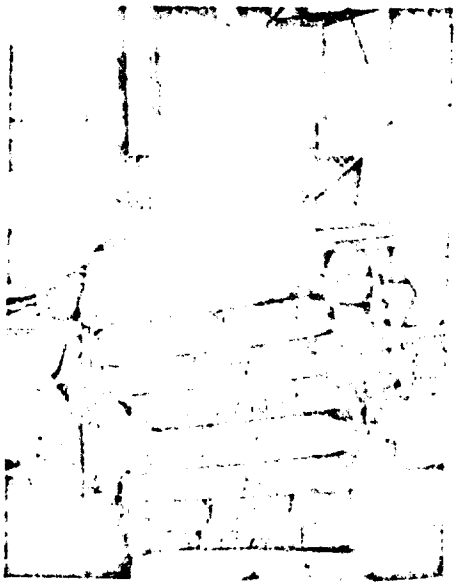
The answer for correcting these compaction problems came to Gold Kist in the form of a sample of Nopak® feed conditioner supplied by the Clay division of J. M. Huber Corp. This is an inert conditioner based on a very fine particle kaolin clay. Huber modifies it and makes the surface of the clay hydrophobic or water repellent.

In actual use, the conditioner is mixed intimately with the sticky soy meal, coating the moist meal particles and preventing them from adhering to one another. Highly improved flow is the result —even under extreme weight loads at the bottom of a filled storage bin or hopper car.

Investigation showed that the most appropriate location to introduce the Huber conditioner into the Gold Kist system was at the end of the meal-cooling operation. At this point, the processed meal contains about 12.5% moisture and, subsequently, will be screened and sized for storage, blending, and shipping.

A hopper for the conditioner, a screw conveyor, and a variable speed drive were the only additional equipment required to feed the conditioner directly into the stream of soy meal discharging from the rotary cooler.

The Gold Kist conditioner system paid for itself in reduced direct costs within 2 months. And this does not take into account potential damage to the bins, increased demurrage for hopper cars, and similar related costs that don't occur when the Huber conditioner is used. ◆



Nopak clay is fed into the soy meal process before screening and storage.

Potts-'53
Bib No. 530

Brit. Jour. Of Surgery 41:295-299. 1953.

KAOLIN GRANULOMA

By R. ERIC POTTS

FROM THE PATHOLOGY LABORATORY, KILMARNOCK

WHILE the occurrence in skin and subcutaneous tissue of granulomata caused by the implantation or impaction of silicious particles is recognized, it has not received the prominence accorded to the analogous lesions of intra-abdominal tissues. In contradistinction to the latter, however, the silicious granulomata of skin and subjacent tissues are not confined to those produced by talcum powder. Thus, Shattock (Shattock and Dudgeon) (1916) reported a local silicious granuloma due to impacted stone many years before talcum-powder granuloma was first recognized by Antopol (1933). It is the purpose of this paper to call attention to the occurrence of a further variety of local silicious foreign-body reaction, namely, kaolin granuloma.

CASE REPORTS

Case 1.—

CLINICAL SUMMARY.—C. W., a nurse aged 22 years, complained of pain in and discharge from the left breast. She stated that sixteen months previously she had developed a painful swelling above and medial to the nipple and that following rupture of this inflammatory mass, a sinus had formed. Kaolin poultices were applied on several occasions during this time but the lesion failed to heal, although temporary improvement occurred during penicillin therapy. A wedge of tissue including the sinus was excised on Feb. 7, 1952. There had been no previous operative treatment and no dusting powder had ever been applied to the area. The patient gave an excellent history.

PATHOLOGY.—The specimen comprised a piece of fibro-fatty tissue 27 × 24 × 16 mm. covered by skin.

Histologically, the subcutaneous tissue was largely replaced by a chronic inflammatory mass. Superficially, this granulomatous lesion extended up to, and at one area ulcerated the covering epithelium, while laterally and deeply it merged into the dense collagenous scarring of the surrounding tissue. In the granulation tissue the major part of the cellular infiltrate was formed by lymphocytes, plasma cells, and large mononuclears, but polymorphonuclears were present at some areas. The most striking feature of the tissue reaction was, however, the presence of moderately-sized multinucleate giant cells in which the irregularly-distributed nuclei seldom exceeded ten in number (*Fig.* 360). These cells, smaller in size but otherwise similar to the foreign-body type of cell commonly encountered, showed small translucent cleft-like spaces in the cytoplasm when examined by direct illumination: crossed polaroid screens revealed these as birefringent crystalline particles. Using these screens it was also apparent that there was a significant correlation between the occurrence of the giant cells and the distribution of the doubly refractile material. The latter was not, however, confined to the multinucleate cells, since a few of the large mononuclears also contained similar particles, but almost all the crystals were intracellularly situated in one or other of these two cell types (*Fig.* 361). The crystals themselves were uncoloured and most frequently of spicular contour, although their small size, which was of the order of 10 microns, precluded a detailed study of their form. All resisted immersion in *N* hydrochloric acid for 30 minutes, and exposure to 600° C. for 10 minutes.

Case 2.—

CLINICAL SUMMARY.—M. M., a 30-year-old housewife, complained of a recurrence of inflammation in the coccygeal region. A pilonidal sinus had been discovered in 1942. She had suffered the usual recurrent inflammatory episodes and had applied kaolin poultices in 1949. The affected area was excised on March 30, 1952.

PATHOLOGY.—The specimen comprised a skin-covered portion of tissue 45 × 20 mm. in surface extent and 15 mm. in depth.

Histologically, this showed a small sinus extending down into the subcutaneous tissue, lined in part by squamous epithelium and in part by chronic non-specific granulation tissue. The dermis and subcutaneous tissue adjacent to the sinus were widely involved by fibrous

scarring, in the mature collagenous tissue of which were scattered cellular foci consisting of lymphocytes, plasma cells, and large mononuclears, together with a variable but generally small number of giant cells. These latter were uniformly of foreign-body type and frequently contained tiny spicular crystals inconspicuous by ordinary illumination, but brightly visualized between crossed polaroid screens (*Fig.* 362). All these crystalline inclusions resisted exposure to *N* hydrochloric acid for 30 minutes and exposure to 600° C. for 10 minutes.

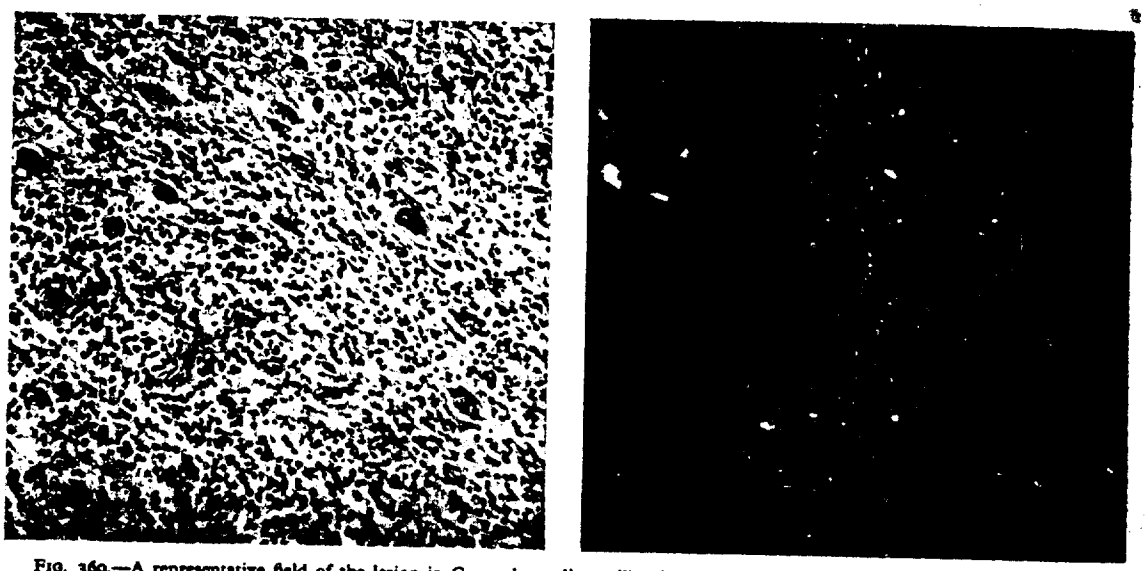


FIG. 360.—A representative field of the lesion in *Case* 1 by ordinary illumination and through partially crossed polaroid screens. In the chronic granulomatous tissue reaction several small multinucleate giant cells are present, some of which are seen to contain birefringent kaolin crystals. Many particles are, however, outwith these giant cells. Hæmatoxylin and eosin. (× 140.)

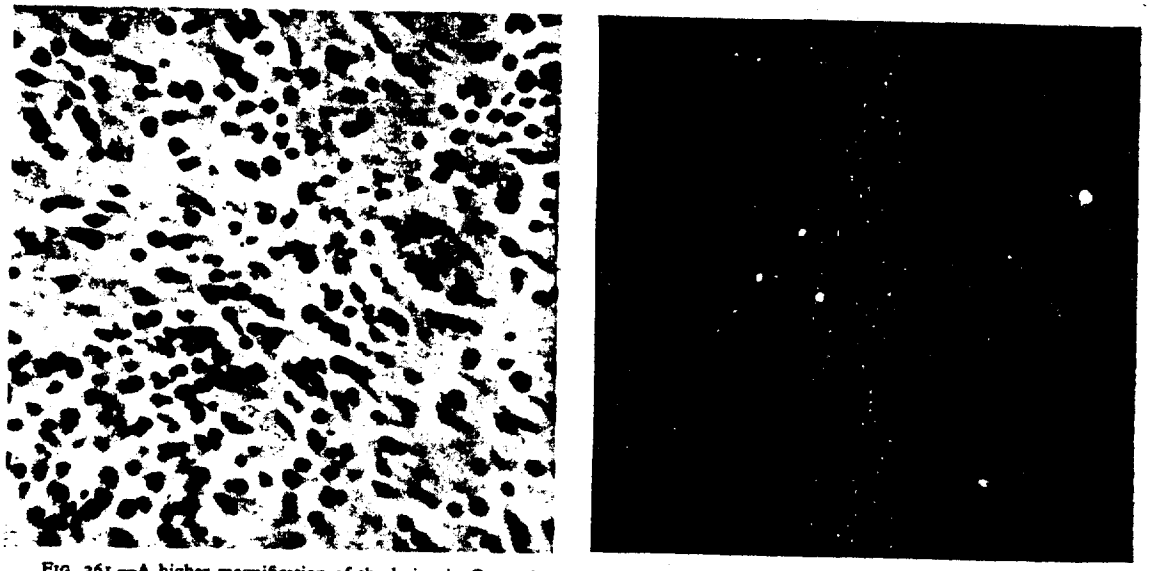


FIG. 361.—A higher magnification of the lesion in *Case* 1 by ordinary illumination and through partially crossed polaroid screens. This shows the intracytoplasmic situation of the majority of the crystals within giant cells or large mononuclears. A number of polymorphonuclear leucocytes are present in this field. Hæmatoxylin and eosin. (× 400.)

DISCUSSION

Commentary on the Cases.—In view of the frequency with which kaolin poultices are used in the treatment of inflammatory diseases of skin and subcutaneous tissue, it is remarkable that the occurrence of local foreign-body granuloma due to this substance has not, so far as I am aware, been reported hitherto. It does not, however, follow that the development of silicious granuloma in such cases is a rarity, for only those lesions which fail to heal after a very long time reach the surgeon, while still fewer are subjected to excision or biopsy and ultimately pass to the pathologist. Moreover, kaolin crystals are inconspicuous in the tissues and are readily overlooked unless the section is examined between crossed polaroid screens, and even if

recognized they are likely to be regarded as talc. The two varieties of crystal are, indeed, very similar in that they are both spicular in form and of the order of only a few microns (*Figs.* 363, 364). Kaolin crystals are somewhat smaller than those of talcum powder but the difference is not sufficient to provide a reliable morphological distinction. Both are colourless by direct illumination and doubly refractile between crossed Nicol's prisms, but it should be mentioned that by no means all the particles in either talcum powder or kaolin powder are birefringent, even when examined at various degrees of rotation on a rotating stage.

It is not surprising that the two types of crystal elicit a similar tissue reaction since both are naturally-occurring insoluble silicates. Talcum powder consists largely of hydrated magnesium silicate ($3MgO.4SiO_2.H_2O$) while kaolin is mostly composed of hydrated aluminium silicate ($Al_2O_3.2SiO_2.2H_2O$).

The cellular reaction in the two cases described is closely similar to that regularly encountered in granulomata due to talcum glove powder, and both were, in fact, initially regarded as instances of talc granuloma until subsequent inquiries excluded the possibility of ingress of such material into the tissues and established at the same time a clear history of repeated applications of kaolin.

The role of the implanted kaolin as a factor in the failure of healing in the second of the two cases is speculative, since chronicity of the inflammation in

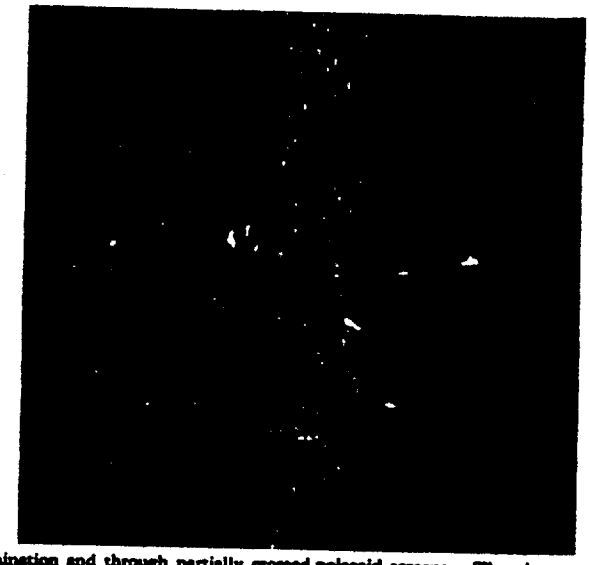


FIG. 362.—The lesion in *Case* 2 photographed by ordinary illumination and through partially crossed polaroid screens. The giant cells are larger than in *Case* 1 and lymphocytes are more conspicuous. Doubly refractile crystals are again seen, both within the multinucleate giant cells and outwith them. Hæmatoxylin and eosin. (× 140.)

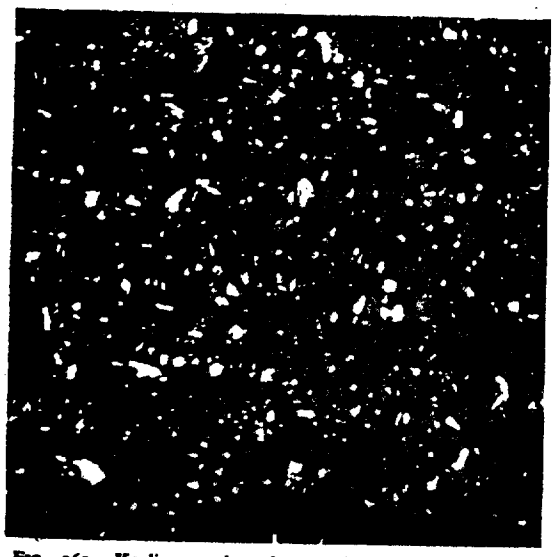


FIG. 363.—Kaolin powder photographed through crossed polaroid screens. (× 140.)

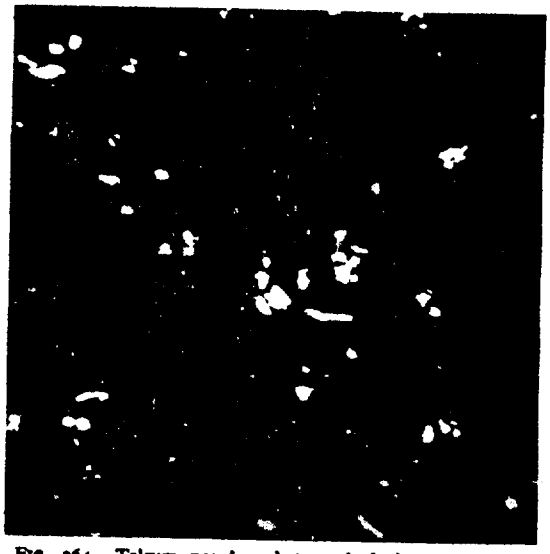


FIG. 364.—Talcum powder photographed through crossed polaroid screens. (× 140.)

pilonidal sinuses is the rule rather than the exception. In the first case, on the other hand, there seems little doubt that the presence of the kaolin crystals and the resultant foreign-body reaction was responsible for the maintenance of the sinus—a view which is consistent with the very temporary improvement during penicillin therapy.

It is known that kaolin gives rise in the tissues to a chronic inflammatory lesion followed by fibrous scarring, and it has been used for the purpose of obliterating the pleural cavity (Baader, 1950). It is also of interest that pneumoconiosis due to long exposure to kaolin dust has been described in two pharmaceutical workers, although the diagnosis was clinical (Tara and Trouard-Riolle, 1948). Nevertheless, I considered it desirable to confirm the granuloma-eliciting property of kaolin by inoculation into the tissues of the experimental animal. Examples of these artificially-produced lesions are shown in *Figs* 365 and 366. They left no doubt of the capacity of kaolin to elicit a foreign-body giant-cell reaction indeed, the early lesions consisted predominantly of multinucleate giant cells with an admixture of large mononuclears, while, in contradistinction to

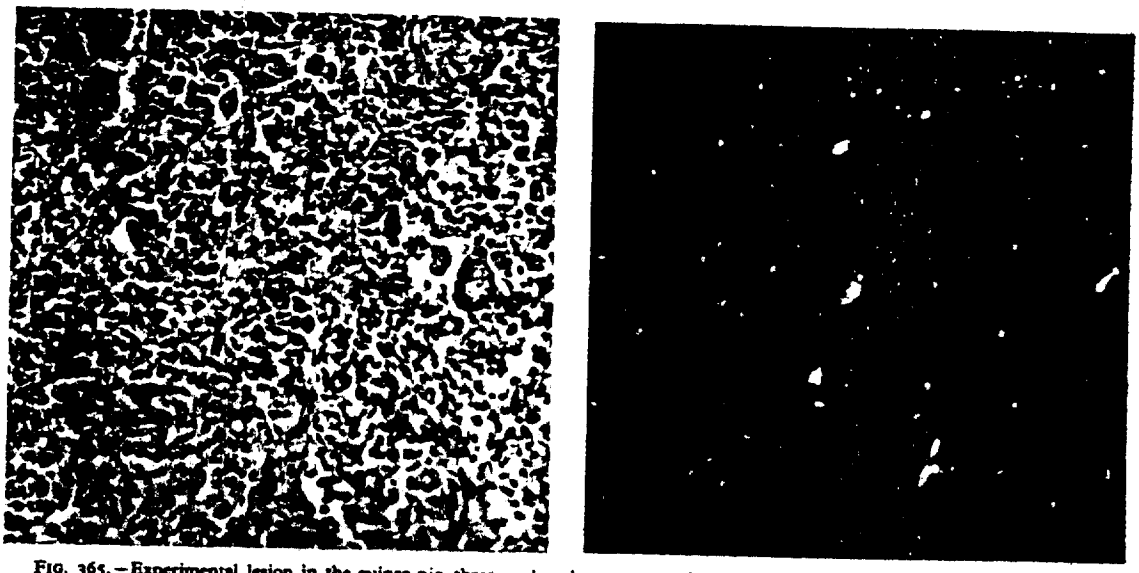


FIG. 365.—Experimental lesion in the guinea-pig, three weeks subsequent to subcutaneous injection of kaolin in aqueous suspension, by ordinary illumination and through partially crossed polaroid screens. The lesion consists almost entirely of binuclear and small multinuclear cells with a smaller proportion of mononuclear histiocytes. The Langhans-like cell on the right-hand side was one of many in this tissue. The crystals are inconspicuous by direct illumination, but are prominent between polaroids. The majority are clearly intracellular and in most instances very small, although several larger particles are also seen. Hæmatoxylin and eosin. (× 140.)

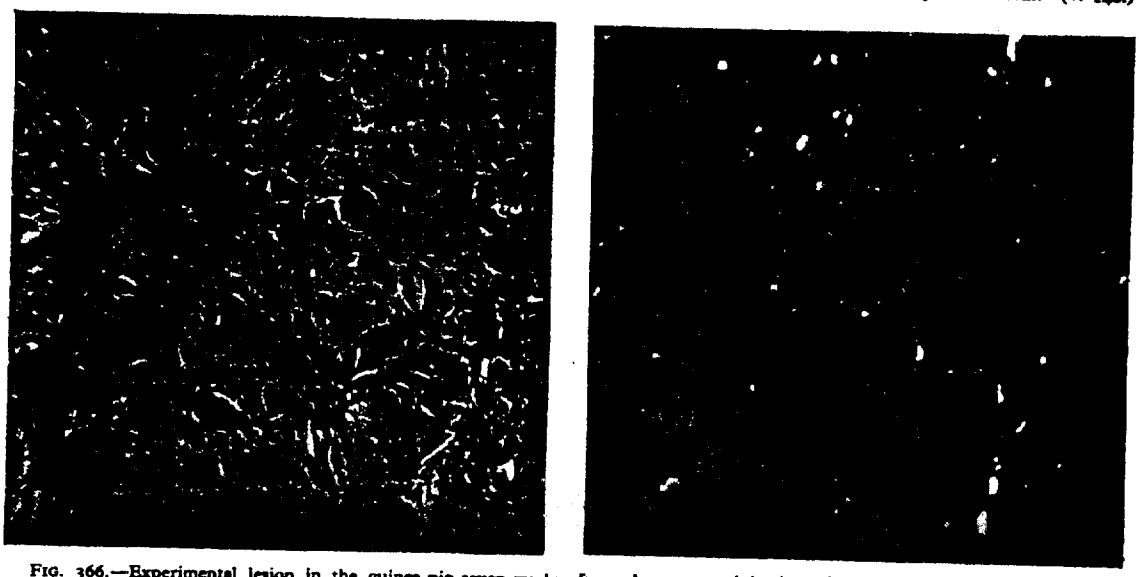


FIG. 366.—Experimental lesion in the guinea-pig seven weeks after subcutaneous injection of aqueous suspension of kaolin, by ordinary illumination and through partially crossed polaroid screens. This shows a similar histological picture to the three-week lesion with the addition of early fibrosis. Iron hæmatoxylin-picro-acid fuchsin. (× 140.)

the human cases, lymphocytes and plasma cells were present in negligible numbers and no granulocytes were found. The experimental granulomata were, however, closed lesions, free from the effects of secondary pyogenic inflammation which existed in the human cases described. There was no significant fibrosis in the earlier lesions, but at seven weeks there was appreciable fibroblastic and, at places, more collagenous scarring. In short, there remained no doubt that kaolin might be responsible for a silicious granuloma very similar to that produced by talc.

Differentiation from Talc Granuloma.—Although in a lesion established as a silicious granuloma the differentiation of kaolin and talc as the aetiological agent may be obtained by careful inquiry, it would be advantageous to have available a simple method for the independent identification of the two types of crystal in tissue sections. Both are highly insoluble substances and resist immersion in mineral acids in all strengths, but at the same time insolubility in the strictly chemical sense is commonly relative rather than absolute, and such is the case with hydrated aluminium silicate. It seemed, therefore, feasible that a sufficient number of aluminium or silicate ions might be available in solution to visualize one or other of these radicles. Alizarin sulphonate is a sensitive although not specific reagent for the detection of aluminium, while the acidified ammonium molybdate-benzidine technique is specific for silicates in the absence of phosphates and with the dismissal of arsenates and germanates. Initial 'spot-test' trials with kaolin powder on filter-paper failed to show any perceptible colour with alizarin Red S in either aqueous or alcoholic solution and this test therefore was not further considered. On the other hand, with the acidified-molybdate method the formation of silico-molybdate from the kaolin was readily visualized by benzidine reduction, while no reaction was obtained with parallel tests using talc, so that the method clearly offered prospects of successful adaptation to the differentiation of the two substances in tissue sections. This expectation was not, however, fulfilled: microscopically there was no appreciable staining of the kaolin crystals in either the human or experimental lesions, despite the fact that in the sections of the more heavily kaolin-impregnated animal tissues a faint but unequivocal blue coloration was evident on naked-eye examination. It is, unfortunately, a serious shortcoming of the very highly sensitive molybdenum blue method that the coloured end-product is subject to considerable diffusion; while ammonium silico-molybdate, unlike the corresponding phospho-molybdate, is soluble in acid. Furthermore, a blue coloration is not readily detected microscopically in small amounts even if the illumination is reduced to take advantage of the Purkinje effect. For the moment, therefore, there appears to be no method, other than by direct inquiry, of identifying as kaolin small amounts of doubly refractile silicious material such as are found in the tissues.

Differentiation from Proliferative Tuberculosis and Sarcoidosis.—In the wider differential diagnosis of kaolin granuloma, it should be borne in mind that talc may give rise to a follicular granuloma bearing a histological resemblance to tuberculosis or sarcoidosis, especially where the quantity introduced into the tissues is small and widely scattered. This similarity is prominent, too, in some cases of traumatic silicious granuloma due to stone particles and, indeed, was responsible for the designation 'pseudo-tuberculoma silicoticum' applied to this type of lesion. It seems likely that kaolin might elicit a similar reaction, and attention is therefore drawn to the fact that intracellular non-silicious doubly refractile crystalline inclusions may occur in both sarcoidosis (Sklavlem and Ritterhoff, 1946) and hyperplastic tuberculosis (Potts, 1951). In this connexion it is of differential diagnostic importance that the endogenous crystals of both tuberculosis and sarcoidosis are always soluble in *N* hydrochloric acid within half an hour, in contradistinction to silicious crystals, whether kaolin or talc.

SUMMARY

Two cases of subcutaneous kaolin granuloma are described. The lesions are morphologically indistinguishable from those caused by talc. Similar lesions, produced by the injection of kaolin in experimental animals, are illustrated.

The chemical differentiation of kaolin and talc crystals was achieved in macroscopic spot tests, but the same method was not successful in tissue sections.

Kaolin, like talc, is resistant to dilute mineral acid, and in consequence is readily differentiated in the tissues from the endogenous birefringent crystalline inclusions of tuberculosis and sarcoidosis.

I am indebted to Dr. W. B. Davis for his helpful suggestions in the preparation of the script. Mr. R. Cormack and Mr. G. Ralston kindly arranged for detailed clinical histories. The photomicrographs are the work of Mr. H. Gray.

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MEDIASTINAL AND SUBCUTANEOUS CERVICAL GRANULOMAS PRODUCED BY FAULTY ESOPHAGEAL INTUBATION OF KAOLIN MIXTURE IN MACAQUES[1,2,3,4]

R. E. REED, MARION G. VALERIO, B. M. ULLAND, D. A. VALERIO, AND J. L. STOOKEY[5]

SUMMARY • *Sixteen macaques from 6 different laboratories developed subcutaneous cervical or mediastinal foreign body granulomas associated with an accidentally introduced kaolinite preparation. Lesions in cervical tissues were, with 1 exception, relatively benign, but in 2 of 6 monkeys with mediastinal involvement there was mild to marked respiratory distress. Elongated and irregularly outlined, highly birefringent crystals observed in all affected tissues were identified as kaolinite. Silica, dickite and carbonate apatite were also present in some tissues. The lesions were deduced to be the result of injury and penetration of the pharynx or cheek pouch by the rigid metal cannulae used to administer antidiarrheal suspensions containing kaolin.*

Granulomatous inflammation caused by many different agents is so commonly observed that it must be associated with a unique or little-understood cause before provoking comment. This was evident in the cases reported here, in which the response of monkey tissue to a foreign material (kaolinite) is described.

Sixteen monkeys from 6 different laboratories were involved. Two devices are commonly employed for gastric intubation of simians (Fig. 1). One is a disposable polyethylene infant feeding tube used for intubation of medication directly into the stomach. The other is a stainless steel rodent stomach tube with which medication is discharged in the posterior pharynx, forcing the animal to swallow. It seems unlikely that the pliable polyethylene feeding tube could actually penetrate the posterior pharyngeal mucosa; therefore, accidental introduction of foreign material into the lung is the main hazard. Conversely, the stainless steel tube could readily puncture the pharynx or perhaps the cheek pouch. The medication, when deposited beneath the mucous membrane of the mouth, could then gravitate to any point along the para-esophageal fascial plane, even into the mediastinum. If such accidents were not observed or reported by animal attendants, circumstances leading to the formation of a lesion would be forgotten.

The presence of kaolinite in monkey tissues was manifested by subcutaneous swellings in the cranial cervical area and by intrathoracic lesions. Histories and gross observations for each reaction will be discussed.

CLINICAL FEATURES

A subcutaneous nodule was noted in the neck region of each of 10 monkeys either at the time of admission to the laboratory, dur-

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[1] Original observations presented at the 18th Annual Meeting, American Association for Laboratory Animal Science, Washington, D. C., October, 1967, by Marion G. Valerio and B. M. Ulland.

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[3] The "Principles of Laboratory Animal Care" as promulgated by the National Society for Medical Research were observed during this study.

[4] Accepted for publication May 4, 1970.

[5] R. E. Reed, Registry of Comparative Pathology, Armed Forces Institute of Pathology, Washington, D. C. Present Address: Department of Animal Pathology, College of Agriculture, University of Arizona, Tucson, Arizona. Marion G. Valerio, B. M. Ulland, and D. A. Valerio, Biometics Research Laboratories, Inc., 7300 Pearl Street, Bethesda, Maryland. J. L. Stookey, Veterinary Pathology Division, Armed Forces Institute of Pathology, Washington, D. C.

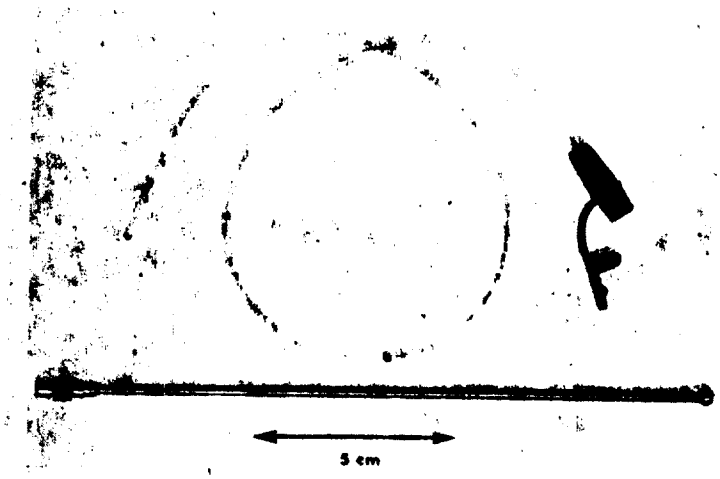

Fig. 1. *Devices used for gastric medication. Top — disposable polyethylene infant stomach tube; bottom — stainless steel rodent stomach tube.*

ing experimental procedures, or at necropsy. This group included 7 *Macaca mulatta*, 2 *Macaca nemestrina*, and 1 *Macaca arctoides*. There was no correlation with age and sex. All nodules, or masses, were located just lateral to the midline somewhere between the larynx and the thoracic inlet. The size varied up to 7 cm in diameter (Fig. 2).

Fig. 2. *Subcutaneous cervical granuloma in a Macaca arctoides. AFIP negative 69-10461.*

Six additional *M. mulatta* were found with intrathoracic lesions. Three of these were detected on routine radiographic studies. Anteroposterior and lateral views of the thorax revealed ventral-lateral displacement of the trachea and esophagus, with an area of increased density in the cranial mediastinum (Fig. 3). Respiratory distress was evident in 2 monkeys, becoming severe in 1 upon handling. With the exception of radiographic observations, all diagnostic procedures and determinations were within normal limits.

Fig. 3. *Radiograph, lateral view, Macaca mulatta. Note the extreme ventral displacement of the trachea and increased density in the cranial mediastinum. AFIP negative 69-10462.*

The other 3 cases were incidental findings at necropsy.

Gross Observations

At the time of necropsy or surgical excision, the subcutaneous nodules varied in gross appearance. They were manifested as 1) small cystic masses located at the angle of the mandible or near the larynx, 2) spongy white masses of abnormal tissue, 3) hard, yellow and lobulated masses on cut surface, or 4) fluctuant masses containing creamy gray, non-odorous, paste-like material (Fig. 4). These occurred anywhere between the mandible and thoracic inlet.

The pertinent necropsy findings in those with intrathoracic lesions were confined to the neck and mediastinum. Thin yellow sheets of tissue reminiscent of thymus or pancreas extended from the anterior larynx along one or both sides of the esophagus and trachea to the thoracic inlet and into the mediastinum, where they became continuous with a large, either firm or fluctuant mass. The mediastinal masses had both cystic and solid components. Cut surfaces of the solid lesions were firm, yellow, and lobular. Incision of the cystic masses exposed a large cavity filled with flocculent, creamy to sanguineous fluid. The capsule was 5-6 mm thick, and trabeculae coursed through the cavity (Fig. 5). The lungs were displaced and compressed laterally (Fig. 6). In 1 of the 6 there was a semifirm mediastinal mass that, upon incising, contained thick, creamy, non-odorous material, which probably represented an earlier lesion. Samples taken for bacterial and mycological cultures and virus-isolation studies yielded negative results.

Fig. 4. *Gross specimen, resected subcutaneous cystic cervical mass from a Macaca arctoides. Top — intact specimen; bottom — specimen with wall incised. AFIP negative 69-9715.*

Histologic Findings

Whether solid or cystic, the principal com-

Fig. 5. *Gross specimen illustrating incised mediastinal lesions in a Macaca mulatta. T = trachea, L = lungs, C = cystic component of granuloma, S = solid granuloma. AFIP negative 68-5900-1.*

Fig. 6. *Photomicrograph of a cross-section through the mediastinal mass and lungs in toto. Note the lateral compression of esophagus, aorta, and lungs. Arrow denotes the cystic center of the granuloma. Hematoxylin and eosin. Approx. 3X. AFIP negative 69-10463.*

Fig. 7. *Photomicrograph of a granuloma under partially polarized light demonstrating birefringent crystals. 440X. AFIP negative 70-2034.*

ponent of the granulomatous lesions in each monkey was formed by aggregations of large mononuclear phagocytes with abundant pink cytoplasm. The macrophages formed solid sheets with very little fibrous tissue stroma or were arranged as walls of considerable depth around cystic spaces containing amorphous eosinophilic granular material and irregularly distributed fibrous strands. Many highly birefringent needle-shaped and irregularly outlined crystals up to 16 μ in length were present in the contents of the cyst and in the macrophages (Fig. 7). Peripheral compression and epithelialization were not features of the reaction except in 1 case. Clusters of macrophages could be seen spreading pre-existing collagen, skeletal muscle fibers, and other normal structures at the periphery of lesions. Reactive fibrosis was minimal, although some degree of encapsulation was present in every case, and the macrophagic masses were irregularly lobulated by fine strands of fibrous tissue.

Except for 1 case in which the subcutaneous nodules extended to the thoracic inlet, there was no necrosis. In this exceptional case only smooth and skeletal muscle and some connective tissue elements of the esophagus were viable in a section taken from the lower neck.

Relatively few inflammatory cells of other types were noted. Some lesions had small foci of lymphocytes or neutrophils associated with vessels penetrating the macrophagic mass or adjacent to cystic spaces. A few spindle-shaped fibroblasts were scattered in the amorphous, granular contents of the cyst. Numerous foreign body giant cells were a striking feature (Fig. 8).

Regional lymph nodes, when present in sections, were depleted of lymphocytes and frequently had clusters of large macrophages containing intracytoplasmic birefringent crystals in the sinuses.

Lung tissue was available for examination from several cases. Macrophages containing birefringent crystals were observed in alveoli and alveolar walls, in the adventitia of blood vessels, perivascular lymphatic spaces, and in

Fig. 8. *Photomicrograph of a granuloma. Note the broad sheet of foamy macrophages and giant cells. Birefringent crystals were found throughout the section. Hematoxylin and eosin. 200X. AFIP negative 69-9780.*

the supporting tissue of bronchioles. The phagocytes formed clusters, but there was no organization analogous to that seen in the subcutaneous cervical or intrathoracic lesions.

While there was no definite fibrous tissue capsule around some of the lesions, mature collagenous tissue strands were prominent among the macrophages in the wall. In 1 case portions of the central granular mass and also the wall were mineralized.

Granular material from the cyst or tissue containing phagocytized crystals from each lesion was digested in sodium hydroxide and subjected to x-ray diffraction by Dr. Frank B. Johnson, Histochemistry Branch, Armed Forces Institute of Pathology (AFIP). Kaolinite was identified in all samples. Material from the mediastinal lesions was also shown to contain silicon and aluminum according to the technics of C. Hozdic (4) and Hashmi *et al* (3) after prior tissue digestion according to methods of I. Bergman (2). Silica, dickite, and carbonate apatite were identified from material of several different granulomas.

Discussion

Kaolin is a form of aluminum silicate familiar to many as China clay. Its medicinal use in combination with pectin and antibiotics is well known. A form of pneumoconiosis associated with inhalation of kaolin dust occurs among workers in the ceramics industry (6). There is little similarity, however, between the foreign body response of simian tissue just described and the reaction of human lung to inhaled kaolin dust. Subcutaneous granulomas have been described in man which were associated with the traumatic introduction of crystalline material containing silica (1). Experimental production of an adhesive arachnoiditis by intracisternal injection of kaolin was reported by McLarnin *et al* (7). The tissue response to kaolin is largely histiocytic and fibroblastic with an occasional minimal lymphocytic focus (6).

The cases described in this report were referred to the Registry of Comparative Pathology of the AFIP within a year's span from 6 different laboratories which indicates widespread occurrence and use of a hazardous technic for gastric intubation of simians. One of the cases involving a subcutaneous granuloma has recently been reported (5). A group of 4 animals had history of medication with a kaolin preparation after receipt by the laboratory. Monkeys involved were part of a colony in which *Shigella* infections were occurring. Although none of these became infected, they may have been treated with antidiarrheal medication upon the observation of nonspecific diarrhea during efforts to bring shigellosis under control. Medication with a syringe and inflexible cannula was common practice at the time and might have led to the forced injection of kaolin suspension into the retropharyngeal tissues.

The finding of focal accumulations of macrophages containing these birefringent crystals in the lung is evidence of some degree of inhalation of the medication. These were not associated with lesions caused by *Pneumonyssus simicola*.

If the traumatic introduction of kaolin medication also accounts for the remaining cases, then the experience of the other laboratories indicates that some macaques are being injured while still in the hands of procurement and supply organizations. Use of flexible infant stomach tubes introduced through the nares as recommended for administering liquid gastrointestinal medication would probably eliminate further production of such lesions.

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Histochemical Changes in the Rat Small Intestine Associated with Enhanced Absorption after High Bulk Feeding

By E. O. Riecken*, R. H. Dowling**, C. C. Booth and A. G. E. Pearse, London

Summary. The dietary stress of high bulk feeding in the rat has been shown to promote increased absorptive capacity by the small intestine.

The histochemical enzyme pattern of the small intestine in rats so stressed was investigated to explore a possible enzyme mediated transport system. This enzyme pattern in the bulk fed animals was compared with the pattern from corresponding intestinal sites in control animals given a normal diet.

The intestine from the rats stressed with kaolin as a dietary bulk expander, showed an increase in the activity of leucine amino-peptidase and in the activity of several dehydrogenases.

The significance of these findings in relation to the enhanced absorptive capacity is discussed.

The response of the small intestine to various dietary stresses has been the subject of many communications. As early as 1903. *Babák* reported an increase in length of the tadpole intestine after feeding vegetable proteins. In rats, intermittent starvation produced a similar response with an increase in both weight and length of the small intestine (*Fabry and Kujalová*, 1959). These workers also found an enhanced absorptive capacity in rats stressed in this way. The similar dietary stress of semistarvation in rats, also produced an enhanced capacity of the small intestine to absorb glucose and histidine (*Kershaw, Neame and Wiseman*, 1960).

Many papers have commented on the morphological response of the gastrointestinal tract to high bulk feeding. Most reports suggest that there is increase in length or hypertrophy of the small bowel in animals fed bulky diets (*Addis*, 1931; *Wierda*, 1950, and *Friedman*, 1953). To explore possible functional changes which may have accompanied

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TABLE I

Morphological response of the small intestine to the stress with kaolin diets

Type of diet	Wet weight	Small intestine histology		
		Villous height μ	Total mucosal thickness μ	Muscle thickness μ
Control	5.04 g	387	580	79
1:2 kaolin	4.93 g	400	500	77
1:4 kaolin	4.99 g	405	575	85

this morphological response, absorption studies have been carried out with the small intestine of rats whose diets were diluted with powdered kaolin and the results are reported elsewhere (*Dowling, Laws and Booth*, 1965).

Our studies failed to demonstrate hypertrophy of the small intestine but absorption studies showed a striking increase in the capacity of the small bowel to transport glucose and tryptophan. These results are illustrated briefly in Table I and II. This increase in absorptive capacity might reflect altered activity in an enzyme mediated system. It was thus the purpose of this investigation to study the histochemical pattern of the small intestinal mucosa after kaolin feeding.

TABLE II

In vitro glucose transport in kaolin fed rats after 9 months of dietary stress. Results are expressed as transport of glucose in mg per 100 mg of dry weight intestine

Type of diet	Glucose transport (mean ± S.D.)
Control	252 ± 228 mg
1:2 kaolin	481 ± 309 mg
1:4 kaolin	996 ± 185 mg

Material and Methods

18 adult female rats from a hooded, laboratory inbred strain were studied. Three different cages were used each containing six animals. Group 1 were fed a standard laboratory diet (diet 41B mash as supplied by Messrs. E. Dixon & Sons, Cranemead Mills, Ware, London), and served as controls. Group 2 received the same food mixed with 2 parts by weight of light powdered kaolin (B.P.) and group 3 was given a 1:4 mixture of standard food and kaolin. The animals were fed *ad libitum* and had free access to tap water. A set of three animals, one from each group was killed at different time intervals after starting the diet. The first set was sacrificed after 2½ weeks, 3 further sets after 4 weeks and the last 2 sets after 12 weeks of dietary stress.

The animals were starved for six hours, weighed and decapitated after stunning by a blow on the head. A fixed and constant time of sacrifice was strictly maintained. After cutting the mesentery along the intestinal wall with sharp scissors, the small intestine was quickly removed and spread on a cork mat. Great care was taken to avoid tension on the gut. Three segments, each 1 cm in length, were removed at 6 and 30 cm intervals from the pylorus and at 10 cm above the ileocecal valve. The segments were placed longitudinally on a piece of cork and were quenched immediately in liquid nitrogen; to facilitate cutting, the tissue was supported by a piece of rat liver and stored in an airtight plastic bag at −70° C, in preparation for histochemical studies. Adjacent segments were also fixed in ice-cold formal-calcium for 16 hours, washed in gum sucrose for 1 to 2 hours at 4° C and frozen in the described manner.

Sections 8 μ thick, were cut on a cold microtome and placed in a Columbia jar either for prefixation and processing or for immediate incubation. Each batch contained sections from 1 or 2 control animals.

The following enzyme reactions were studied:

Membrane Enzymes

(1) Unspecific alkaline phosphatase (*Burstone*, 1958): substrate naphthol-ASMX-phosphate; diazo-salt Fast Red TR: Cold acetone prefixed sections (45 min) incubated for 10 min at 37° C, alternatively formalin-calcium-fixed sections for 30 min at 37° C.

(2) Adenosine triphosphatase (ATP-ase) (*Wachstein and Meisel*, 1957) at pH 7.2; incubation for 25 min at 37° C, using cold acetone prefixed (45 min) or formalin-calcium-fixed sections.

(3) Amino-peptidase (LAP) (*Nachlas, Crawford and Seligman*, 1957); substrate L-leucyl-4-methoxy-beta-naphthylamide; diazo-salt Fast Blue B; unfixed cryostat sections incubated at 37° C for 10 min.

Lysosomal Enzymes

(1) Unspecific acid phosphatase (*Barka and Anderson*, 1962) at pH 5; substrate naphthol-ASBI-phosphate; coupler hexazonium pararosaniline: cold acetone prefixed sections (45 min) incubated for 25 min at 37° C, alternatively formalin-calcium-fixed sections incubated for 55 min at 37° C.

(2) Glucosaminidase (*Pugh and Walker*, 1958); substrate alpha-naphthyl-N-acetyl-beta-D-glucosaminide: diazo-salt Fast Garnet GBC: cold formalin-calcium-prefixed sections (30 min) incubated for 30 min at 37° C.

Microsomal Enzymes

(1) Unspecific esterase (*Davis*, 1959; *Davis and Ornstein*, 1959); substrate alpha-naphthyl-acetate; coupler hexazonium pararosaniline; cold formalin-calcium-pre-fixed (30 min) or formalin-calcium-fixed (16 hours) sections incubated for 3 min at 18° C.

(2) Glucose-6-phosphatase (*Wachstein and Meisel*, 1956); unfixed cryostat sections incubated for 30 min at 37° C.

Mitochondrial Enzymes

(1) NADH-Diaphorase (*Scarpelli, Hess and Pearse*, 1958); electron acceptor TNBT: cold formalin-calcium-fixed sections incubated for 15 min at 37° C.

(2) Succinodehydrogenase and the following coenzyme-linked dehydrogenases (malate, alpha-glycerophosphate, lactase, and beta-hydroxy-butyrate) were studied by the cobalt-chelating method given by *Pearse* (1958) using MTT as electron acceptor. In addition most of the reactions were also processed using the method of *Nachlas, Tsou, Souza, Chang and Seligman* (1958), using TNBT and NBT; these included the reactions for alcohol and glutamate dehydrogenases which demand KCN as trapping agent. This is incompatable with the cobalt of the MTT method Incubation time of the unfixed cryostat sections was slightly longer when using MTT (50 min, 37° C) than after use of TNBT or NBT (40 min, 37° C).

(3) Monoamine oxidase (MAO) (*Glenner, Burtner and Brown*, 1957); substrate tryptamine hydrochloride; electron-acceptor TNBT; unfixed cryostat sections incubated for 40 min at 37° C.

As a rule, all sections were mounted in glycerine jelly. If possible, as in the case of the hexazonium pararosaniline-coupling methods, they were dehydrated, cleared and mounted in Clearmount *(Gurr)*.

Results

The Normal Enzyme Distribution in the Rat Small Intestine

The strongest activity in most of the enzymes was found in the normal villous epithelium with a gradient from maximum concentration in the upper small intestine to a minimum in the ileum. The epithelial activity usually decreased or completely vanished in the mouths of the crypts. The membrane enzymes were abundant in the brushborder and, in addition, alkaline phosphatase stained the Golgi-area of the upper small intestine. The leucocytes, macrophages and capillaries of the lamina propria also showed alkaline phosphatase activity. ATP-ase gave very much the same pattern as alkaline phosphatase but there was a much stronger reaction in the stroma. LAP was restricted to the brushborder and vanished abruptly at the junction area between the mouth of the crypt and the base of the villus. Non-specific acid phosphatase (lysosomal activity) was mainly confined to granules in the Golgi-area. The positive reaction in the brush-

border of the upper small intestine was partly inhibited by 0.01 in sodium fluoride. Paneth cells and macrophages were also acid phosphatase positive. Glucosaminidase, another lysosomal enzyme, was studied with a technique giving inadequate localization on a cytological level. The epithelium was mainly stained in the apical part, sometimes in the Golgi-area. There was a strong reaction in the lamina propria, probably marking the macrophages and the Paneth cells were also positive. The microsomal enzyme non-specific esterase showed strong activity in the mucous epithelium, maximally in the apical area and lessening in intensity towards the nuclear zone. After a longer incubation-time Paneth cells and macrophages were strongly reactive and this activity was resistant to E600 (diethyl-p-nitrophenylphosphate) indicating its lysosomal nature. The other microsomal enzyme which was demonstrated (glucose-6-phosphatase), was weakly active in the epithelium of the upper small bowel with even less or no activity in the ileum. The strong activity in the brushborder was probably caused by activity of non-specific alkaline phosphatase.

Succinodehydrogenase, used as a mitochondrial marker, showed maximal intensity in both supra- and infranuclear regions. The coenzyme-linked dehydrogenases, as well as the NADH-diaphorase showed very much the same distribution pattern. These enzymes again showed a definite decrease in the crypts. The Paneth cells also stained weakly. The lamina propria showed an overall staining which was less marked than that of the epithelium cells.

MAO gave very much the same localization but the Paneth cells behaved differently, being quite strongly positive with this enzyme.

The use of different electron acceptors gave essentially comparable results.

Histochemical Findings in Kaolin-fed Animals

The results in the bulk fed animals are shown graphically in Table III. An increase in activity when compared with control rats, was definite only in the case of LAP and some of the oxidative enzymes. Eight animals showed an increase of LAP activity and in five of these the increase was present in 2 or 3 intestinal sites (compare Fig. 1a and b). This change in activity was not confined exclusively to the upper or lower small intestine and the intensity of the change did not differentiate between the two different kaolin diets.

The oxidative enzymes showed a similar but less constant increase in activity; however, when present the changes were distinct (com-

TABLE III

Histochemical response to stress with kaolin diets in comparable small intestinal sites. Changes at varying time intervals in enzyme activity as compared with control rats are shown (+ = increase in activity)

Duration of dietary stress	Type of diet	Site of sample from intestine	Alk. P.	A.T.P.	L.A.P.	Ac. P.	Est.	G.-6-P.	N.A.D.H.	S.D.H.	M.D.H.	L.D.H.	Glyc. D.H.	Ale. D.H.	Glu. D.H.	B Hyd. B.D.H.
2½ weeks	1:2 kaolin	Duodenum			+											
		Jejunum			+									+		
		Ileum			+					+				+		
	1:4 kaolin	Duodenum			+											
		Jejunum			+							+				
		Ileum			+					+	+			+		
											+			+		
4 weeks	1:2 kaolin	Duodenum			+											
		Jejunum			+								+	+		
		Ileum			+					+		+	+	+		
	1:4 kaolin	Duodenum			+					+			+	+	+	
		Jejunum			+					+			+	+	+	
		Ileum			+					+		+	+	+	+	
										+			+	+	+	
4 weeks	1:2 kaolin	Duodenum														
		Jejunum								+	+	+				
		Ileum			+						+	+				
	1:4 kaolin	Duodenum							+	+		+				
		Jejunum									+	+				
		Ileum			+						+	+				
					+				+			+		+		

4 weeks	1:2 kaolin	Duodenum	+	
		Jejunum		
		Ileum		+
	1:4 kaolin	Duodenum		
		Jejunum		
		Ileum	+	+
12 weeks	1:2 kaolin	Duodenum		
		Jejunum		
		Ileum		+
	1:4 kaolin	Duodenum		
		Jejunum		
		Ileum		+
12 weeks	1:2 kaolin	Duodenum		
		Jejunum		+
		Ileum		+
	1:4 kaolin	Duodenum		
		Jejunum		+
		Ileum		+

Alk. P. = Alkaline phosphatase
A.T.P. = Adenosine triphosphatase
L.A.P. = Leucino-amino peptidase
Ac. P. = Acid phosphatase
Est. = Non-specific esterase
G.-6-P. = Glucose-6-phosphate
N.A.D.H. = N.A.D.H. diaphorase

S.D.H. = Succinodehydrogenase
M.D.H. = Molic dehydrogenase
L.D.H. = Lactic dehydrogenase
Glyc. D.H. = Glycerophosphate dehydrogenase
Alc. D.H. = Alcohol dehydrogenase
Glu. D.H. = Glutamic dehydrogenase
β Hyd. B.D.H. = Beta hydroxy butyrate dehydrogenase

1a

1b

Fig. 1. Leucine amino-peptidase activity in jejunum. Sections from corresponding sites incubated simultaneously after two and a half weeks of dietary stress. Increased activity is seen in the brush border of the 1 : 4 kaolin fed rat (a) as compared to control animal (b). ×100.

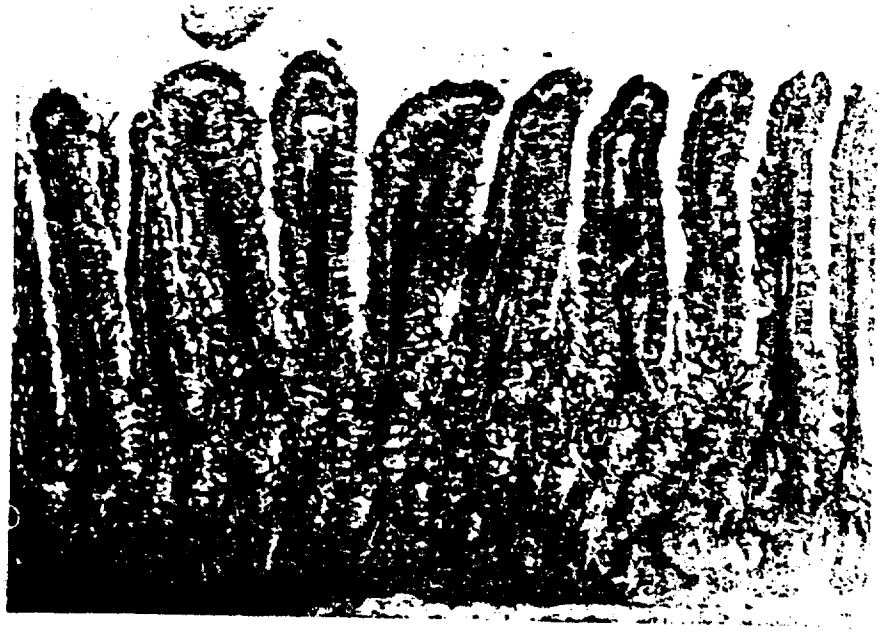

Fig. 2a

pare Fig. 2a and b). Again these enzyme changes could not differentiate the two kaolin diets. A decrease of activity was never observed in the bulk-fed rats when compared with control animals.

Reference to Table III shows that most of the observed changes occurred during the early stages of bulk feeding. Such changes were found between two and a half and four weeks and with the exception of alcohol dehydrogenase were absent at three months after starting the diet.

Discussion

In evaluating the role of enzyme activity in bulk feeding experiments, one must consider the more fundamental relationship between intestinal transport and enzyme activity. In spite of much experimental work, this basic question has not been completely answered. However, there are several weighty arguments which favour the importance of tissue enzymes in intestinal absorption.

There have been many recent reports of disaccharidase deficiency in association with intolerance and malabsorption of the corresponding sugars. Probably the best known example of this, is lactose intolerance particularly in children but also in adults associated with

Fig. 2b

Fig. 2. Glutamic dehydrogenase in jejunum. After four weeks of high bulk feeding increased activity is seen in the absorptive epithelium and lamina propria of the 1:4 kaolin fed rat (a) when compared with control (b). Sections simultaneously incubated from corresponding intestinal sites. ×100.

lactase deficiency of the intestinal mucosa (*Dalqvist*, 1959: *Weijers, van der Kamer and Mossel*, 1960). Inborn metabolic errors are similarly responsible for malabsorption of tryptophan in *Hartnup's* disease, while cystinuric patients lack the aminoacid pump for cystine, lysine, arginine and ornithine (*McCarthy, Borland, Lynch, Owen and Tyor*, 1964; *Thier, Fox, Segal and Rosenberg*, 1964).

Further evidence using histochemical techniques is available from studies using non-specific alkaline phosphatase and ATP-ase. The site of these enzymes is in the brush border of the intestinal villi: in other words the site of active absorption. Furthermore, the concentration of alkaline phosphatase in these sites increases after protein or lipid feeding in contrast to the mucosa in the fasting state (*Tuba*

and Robinson, 1953; *Tuba and Dickie*, 1955). *Moog* (1962) found that alkaline phosphatase activity in the intestinal brushborder of the developmental mouse reached concentration peaks at birth and at the time of weaning. This "supply and demand" relationship suggests that the enzyme may be involved in intestinal transport.

Support for the role of enzyme activity in the increased intestinal transport of bulk fed rats, comes from other experimental work. *Lojda and Fabry* (1959) showed that intermittent starvation in rats caused an increase of hydrolytic enzymes in the intestinal epithelium. *Fabry and Kujalová* (1960) quote previous work from their laboratory demonstrating that rats intermittently starved showed enhanced glucose and fat absorption by the intestine (*Holeckovâ and Fabry*, 1959; *Kujalová and Fabry*, 1958).

An increase in β-galactosidase activity has also been demonstrated in rats after feeding a diet containing 25% lactose (*Fischer*, 1957).

We have demonstrated some enzyme changes in rats stressed with kaolin feeding. Could these changes represent a direct effect of the supposedly "inert" bulk expander? Light powdered kaolin (BP) is purified native aluminium silicate free from gritty particles (*Martindale*, 1958). It does not produce intestinal irritation and it is assumed that no absorption takes place during intestinal transit. However, *Barrnett* (1959), has shown that particulate matter in the form of azo-salts can be absorbed by pinocytosis. If this were so in kaoling feeding, one might expect to find changes in unspecific acid phosphatase activity (a lysosomal marker) which was not present in the intestinal mucosa of our stressed rats. We may conclude that kaolin was not absorbed in amounts sufficient to interfere with staining techniques, if in fact there was any absorption at all.

With respect to the histochemical techniques used, one should perhaps point out that localization of the coenzyme linked oxidative enzymes is determined by the localization and concentration of the diaphorase, for which the reduced coenzyme acts as a substrate.

Previous reports showing "compensatory" increases in intestinal transport in response to dietary stresses suggest two possible mechanisms. First, increase in the absorptive surface area due to villous hypertrophy (*Addis*, 1932; *Kennedy*, 1950; *Wierda*, 1950; *Friedman*, 1953, *Fell, Campbell and Smith*, 1963). As has been shown (*Dowling et al.*, 1965) and in Table I we failed to find any hypertrophy. In addition, had there been an increase in mucosal growth, one might, have been expected a corresponding increase in alkaline and acid phos-

phatase activity of the lamina propria. We failed to demonstrate such a change in the kaolin fed rats and therefore conclude hypertrophy is not the mechanism for increased transport. This is in contrast to our findings in the hypertrophied intestine following intestinal resection in the rat. Here the hypertrophied tissue did show an increase in alkaline and acid phosphatase activity (*Riecken, Dowling, Booth and Pearse*, 1965).

The second known mechanism for increased transport is an increase in an enzyme mediated system. *Lojda and Fabry* (1959) demonstrated an increase in several hydrolytic enzymes in the intestine of rats starved intermittently. Reference to Table III shows increases in LAP activity in 8 out of 12 animals studied following bulk feeding. These changes were definite, though in most instances were less striking than the changes illustrated in Fig. 1a and b. However, it is not known whether or not subtle changes in enzyme activity may produce great or little increase in absorptive capacity.

It will also be noted that there were increases in the staining intensity of various dehydrogenases (Table III). While these changes were not constantly found, it is important to note that where there was an alteration in enzyme activity it was always an increase and never a decrease in the dietary stressed rats.

While these two mechanisms are the only proven ways in which absorption may be enhanced, one may speculate about the role of increased intestinal blood supply or decreased bowel transit time. We have already studied transit time of barium in the small bowel and have demonstrated no change in the bulkfed as compared with the control rats. No information is available about the intestinal blood supply but there was no gross evidence of alteration macroscopically or on histology in the kaolin fed animals.

As mentioned in the results section, the majority of the enzyme increases were found in the first few weeks after starting the diet. We have already shown that this roughly corresponds to an adaptive period before the kaolin-fed rats began to increase their body weights (*Dowling et al.*, 1965). We cannot exclude that the observed enzyme changes reflect a response to adaption. However, the absorption studies did suggest that the progressive increase in transport capacity from control to 1:4 kaolin-fed animals had developed after four weeks.

In conclusion, various enzyme increases have been found in the intestinal mucosa of rats whose diets were diluted with powdered kaolin. These enzymes changes were associated with an increased in-

testinal absorptive capacity but the exact significance of the altered enzyme activity remains uncertain.

However, in the absence of other known mechanisms for enhanced intestinal absorption, it is tempting to postulate that there may be an association between these two findings.

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Authors' address: Dr. E. O. Riecken, Dr. R. H. Dowling, Dr. C. C. Booth and Prof. A. G. E. Pearse, Department of Pathology, Postgraduate Medical School, Ducane Road, *London W. 12* (England)

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X-Ray Diffraction Analysis for Identification of Kaolin NF and Bentonite USP

FARID SADIK, JULIAN H. FINCHER, and CHARLES W. HARTMAN*

Abstract ☐ The identities of kaolin NF and bentonite USP were tested by using the identification test stated in the NF XIII monograph on kaolin. The results were inconclusive because both clays gave positive results to the test. Since kaolin NF and bentonite USP are crystalline in nature, X-ray diffraction analysis could be used for their identification. Because of its accuracy, X-ray diffraction analysis is recommended to be included in the NF and USP monographs on kaolin and bentonite, respectively, as an alternative test.

Keyphrases ☐ Kaolin—identification ☐ Bentonite—identification ☐ X-ray diffractometry—identification, kaolin, bentonite

Kaolin was first introduced into the USP VIII in 1905 as a constituent of Cataplasma Kaolini. Later, it was deleted from the USP IX and was introduced into the NF IV in 1916. Bentonite was first introduced into the USP XII in 1942. The NF XIII monograph on kaolin includes a specific identification test, which is merely a test for the presence of aluminum. On the other hand, the USP XVIII monograph on bentonite does not include a specific identification test but relies on tests such as gel formation and swelling power.

Since both clays are chemically similar and contain aluminum, it was obvious that methods other than the chemical analysis for aluminum needed to be included in the NF monograph on kaolin in order to distinguish between kaolin NF and bentonite USP. Furthermore, it was felt that a specific identification test should be included in the USP monograph on bentonite. The objective of this study was to show the importance of the inclusion of X-ray diffraction analysis in the NF and USP monographs on kaolin and bentonite, respectively. Since both clays are crystalline in nature (1), it was possible to utilize X-ray diffraction analysis to measure the angles of diffraction of the X-ray from the atomic planes of the crystal.

EXPERIMENTAL

NF XIII Identification Test for Kaolin—The NF XIII identification test for kaolin was conducted on kaolin NF and bentonite USP. Both clays responded positively to the test for aluminum, *i.e.*, a gelatinous, white precipitate was produced.

X-Ray Diffraction Analysis—Two types of samples were prepared. An unoriented powder sample was prepared by packing the powder in an aluminum planchet. The powder was smoothed by

Table I—Basal (001) and Higher Order X-Ray Diffraction Spacings of Official Clays[a]

Kaolin NF	Bentonite USP	Glycolated Bentonite USP
7.1091	12.2670	16.2910
4.4445	4.4357	8.3544
4.3496	3.1163	5.6041
4.1639		4.4226
3.8176		4.2108
3.7169		3.3706
3.5587		
3.3731		

[a] Lattice spacings in angstroms.

using the flat side of a spatula to obtain a uniform level suitable to the X-ray beam. A sample of an oriented aggregate was prepared as follows: a 5% (w/w) aqueous clay suspension was prepared by sprinkling the clay over the surface of the water. The mixture was allowed to hydrate for 2 hr. and then was passed through a manual homogenizer. By means of a pipet, 2 ml. of the resultant suspension was withdrawn and allowed to drain onto a glass microscopic slide (27 × 46 mm.) which was completely covered with suspension. The slide was allowed to stand undisturbed at 22.2° (72°F.) and at a relative humidity of 52–55% for 72 hr. to permit air drying.

A Norelco X-ray diffractometer was used. Each sample was placed in the diffractometer and was exposed to a monochromatic X-ray beam generated by using a copper target X-ray tube and nickel filter operated at 40 kv. and 30 mamp. Scanning was begun at 2° (2θ) at the rate of 1°/min. The X-ray diffractogram obtained was recorded by a chart recorder. The sample was then transferred to a desiccator, in which an evaporating dish filled with ethylene glycol was placed. After exposing the sample to the ethylene glycol vapor for 24 hr., a second X-ray diffractogram was obtained as before. Table I shows the basal (001) spacings as well as the spacings of higher order X-ray reflections of each sample.

RESULTS AND DISCUSSION

The NF XIII defines kaolin as a native hydrated aluminum silicate; the USP XVIII defines bentonite as a native colloidal, hydrated aluminum silicate. Thus, the primary elements of kaolin and bentonite composition are aluminum and silicon. Because they contain aluminum in their structure, both clays gave positive results to the identification test of the NF XIII monograph on kaolin, which is basically a test for the presence of aluminum. This finding indicates that the identification test of the NF XIII is inconclusive. To distinguish between kaolin and bentonite, one needs to check other specifications in the NF and USP. The USP XVIII monograph on bentonite does not include a specific identification test.

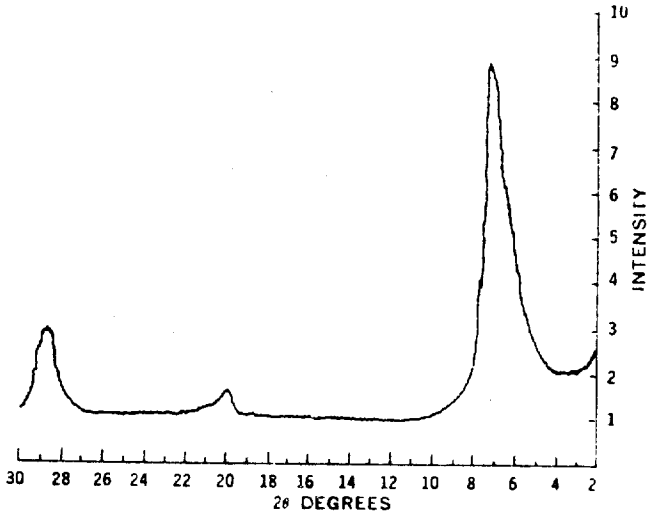

Figure 2—*X-ray diffractogram for bentonite USP.*

Although chemically similar, kaolin NF and bentonite USP differ mainly in their crystal structure. The unit cell structure of kaolin has two layers, stacked one over the other, with their flat sides together. One layer is composed of Al_2O_3 molecules and the other of SiO_2 molecules. The unit cell structure of bentonite is composed of three layers: two layers of SiO_2 molecules with one layer of Al_2O_3 molecules lying between them. This dissimilar assembly of the atoms permitted distinct identification of kaolin and bentonite by means of X-ray diffraction analysis. X-ray diffractograms for kaolin NF and bentonite USP are shown in Figs. 1 and 2, respectively. It is evident that these diffractograms differ and that each clay exhibits a characteristic series of X-ray diffraction peaks.

Because the tests of the official compendia are time consuming and obviously not conclusive, X-ray diffraction analysis was chosen as a methodology which could be conveniently and rapidly used for the identification of kaolin and bentonite. A total time of approximately 40 min. is needed to prepare an unoriented powder sample, obtain an X-ray diffractogram, and identify a sample. The purpose of exposing the clay samples to ethylene glycol is to provide added evidence as to the identity of bentonite and to distinguish it from kaolin. Bentonite is characterized by an expanding lattice. It was shown by MacEwan (2, 3) and MacKenzie (4) that ethylene glycol is adsorbed on the basal plane surface of bentonite and that the C-axis spacing is increased. Ethylene glycol, however, did not affect the nonexpanding lattice of kaolin and the basal plane remained unchanged. The basal plane of glycolated bentonite USP expanded from 7.20° (2θ) to 5.42° (2θ); kaolin NF, when glycolated, remained unchanged at 12.44° (2θ) (Figs. 1–3).

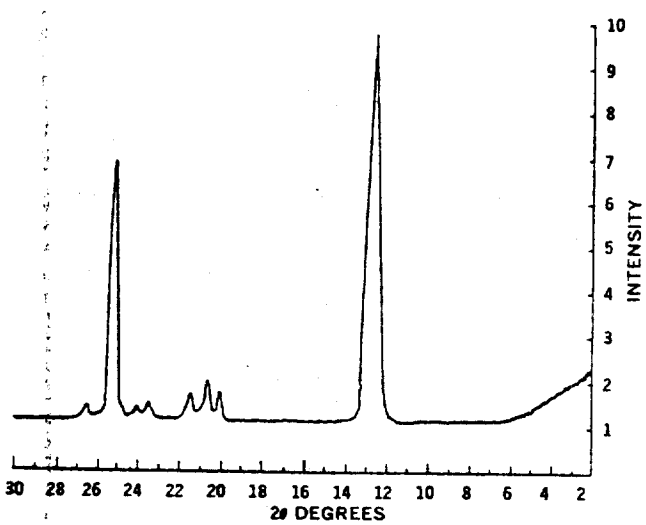

Figure 1—*X-ray diffractogram for kaolin NF.*

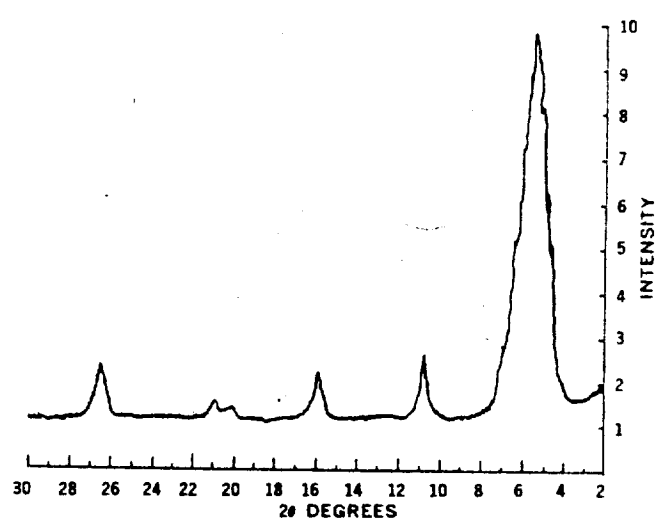

Figure 3—*X-ray diffractogram for glycolated bentonite USP.*

In conclusion, for identification of kaolin and bentonite, X-ray diffraction analysis was found to be more accurate, dependable, conclusive, and much less time consuming than the methods of identification presently employed in the NF XIII and USP XVIII monographs on kaolin and bentonite, respectively. Consequently, it is recommended that these monographs should be supplemented by X-ray diffraction analysis.

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* Deceased.

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The Weight Gain and Feed Intake of Chicks Fed a Ration Diluted with Cellulose or Kaolin

I. R. SIBBALD, S. J. SLINGER AND G. C. ASHTON
Departments of Nutrition, Poultry Husbandry and Physics (Statistics), Ontario Agricultural College, Guelph, Ontario, Canada

The literature pertaining to the effects of the dilution of poultry diets is primarily concerned with experiments in which a relatively inert material was used to replace a more available ration component. This procedure changes the relative proportions of the individual nutrients within the diet and provides little information regarding the properties of the diluent *per se*. There is a scarcity of information concerning the effects of including an inert diluent at the expense of a portion of the entire diet. This latter technique, assuming the diluent to be nutritionally unavailable, should lower the concentrations of the nutrients in the feed without changing their relative proportions. Experiments employing this latter method of dilution should provide useful information not only regarding the properties of the diluents but also concerning the importance of dietary nutrient concentration.

Hill and Dansky ('54) reported that when 10% increments of a 20% protein chick diet were replaced with oat hulls, up to a maximum of 50%, growth and energy consumption decreased while feed consumption increased, although the increased consumption was not sufficient to compensate for the reduced energy content of the diets. An exception to these findings may be noted in the data relating to the treatment group receiving a ration composed of 90% basal diet plus 10% oat hulls; the birds of this group made slightly larger weight gains and consumed more energy than the birds of the control group which were fed the basal diet with no added oat hulls. A comparison of the data obtained when dilution by whole ration replacement was employed with that obtained when dilution was achieved by replacing corn and wheat with oat hulls led the authors to conclude that protein rather than energy was the limiting deficiency. The energy values used were based on the productive energy data of Fraps ('46).

Rand et al. ('56) fed chicks purified diets of varying protein content each supplemented with zero, 10 and 19% of added fiber. No differences in weight gains could be detected between the birds fed the control diets and those receiving the control diets plus 10% of fiber. Nineteen per cent of added fiber reduced weight gains. These findings support those of Hill and Dansky ('54) who demonstrated no depression in growth until oat hulls in excess of 10% were included in the ration at the expense of the basal portion.

Mraz et al. ('57) formulated a series of diets differing in calculated productive energy content. The densities of the diets were varied, by interchanging sand and vermiculite, allowing the authors to study the importance of dietary energy concentration both in terms of calories per unit of weight and calories per unit of volume. The latter measure appeared to be the most useful and it was reported that whereas a productive energy concentration of 0.75 Cal./cm³ was adequate for the maintenance of rapid growth, 0.72 Cal./cm³ was indequate.

The present report concerns an experiment designed to study the influences of cellulose and kaolin upon the feed consumption and weight gains of chicks.

EXPERIMENTAL

The experimental design was that of a randomized block with 4 replicates. Day-old chicks were placed in battery brooders where they were fed the basal diet (table 1) ad libitum. When the birds were two weeks and 4 days old they were weighed, divided into groups having a weight spread

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of 5 gm, and then distributed among 60 pens until each pen contained 14 birds (7 ♂ + 7 ♀ per pen in replicate 1, and 8 ♂ + 6 ♀ in replicates 2, 3 and 4). Each of the 15 experimental diets (table 2) was then randomly assigned to 4 pens. The diet treatments were designed so that increasing amounts of the basal diet (zero, 6, 12, 18, 24, 30, 36 and 42%) were replaced with non-nutritive cellulose,[1] and with kaolin. Following a three-day ration adjustment period the birds entered upon a 7-day experimental period during which weight changes and feed consumption, on a per pen basis, were recorded.

TABLE 1

Composition of the basal diet

	pounds
Ground corn (fine)	36
Ground wheat (fine)	15
Soybean oil meal (44% protein)	32
Stabilized tallow	5
Dehydrated alfalfa meal (17% protein)	2
Meat meal (50% protein)	2.5
Fish meal (65% protein)	2.5
Dried whey product (55% lactose)	2.5
Ground limestone	1.5
Dicalcium phosphate	0.5
Iodized salt (0.015% KI)	0.25
	gm
Manganese sulphate (75%)	9.0
Zinc oxide (80%)	3.0
Vitamin A supplement (10,000 IU/gm)	22.7
Vitamin D_3 supplement (1,500 ICU/gm)	22.7
DL-Methionine (feed grade)	22.7
Riboflavin (1 gm/oz)	5.0
D-Ca pantothenate (2 gm/oz)	5.0
Niacin	0.9
Procaine penicillin G (10 gm/pound)	22.7
Vitamin B_{12} supplement (9 mg/pound)	34.1
Choline chloride (25%)	34.1
3-Nitro, 4-hydroxy-phenylarsonic acid (10%)	22.7

Metabolizable energy (ME) values were obtained for the diets and have been discussed in an earlier report (Sibbald et al., '61a). Ration density values were obtained by two procedures. The first method which employed a device used for the determination of bushel weights of grains yielded density data expressed in terms of grams per milliliter of dry feed. These data (table 2) were considered to be unsatisfactory; not only were they difficult to reproduce but is was also observed that, as the level of dilution with kaolin (diets 9 to 15) increased, the changes in ration density were not linear. The second procedure involved placing 50 gm of feed into a 250-ml volumetric flask, the addition of water from a burette to bring to volume, and frequent shaking to minimize the displacement of water by air. The resulting data (table 2),

[1] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

TABLE 2

Composition and analysis of the experimental diets

Diet	Basal diet[1]	Cellulose[2]	Kaolin	Protein[3]	ME[4]	Density (dry basis)	Density (water displacement)
	%	%	%	%	*Cal./gm*	*gm/ml*	*gm/ml*
1	100	—	—	23.5	2.95	0.574	1.416
2	94	6	—	22.8	2.80	0.546	1.425
3	88	12	—	20.7	2.63	0.521	1.437
4	82	18	—	19.5	2.44	0.505	1.445
5	76	24	—	17.6	2.30	0.458	1.458
6	70	30	—	16.5	2.11	0.439	1.464
7	64	36	—	15.1	1.91	0.411	1.475
8	58	42	—	13.9	1.76	0.378	1.482
9	94	—	6	22.0	2.79	0.622	1.484
10	88	—	12	20.4	2.61	0.695	1.544
11	82	—	18	18.9	2.43	0.677	1.611
12	76	—	24	17.7	2.30	0.658	1.636
13	70	—	30	15.4	2.18	0.625	1.695
14	64	—	36	15.2	1.97	0.588	1.730
15	58	—	42	13.9	1.82	0.676	1.773

[1] Composition of basal diet presented in table 1.
[2] Non-nutritive cellulose, Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[3] Determined by the method of Kjeldahl (A.O.A.C., '55).
[4] Determined by the method described by Sibbald et al. ('61b).

expressed as the weight of feed required to displace 1 ml of water, were considered to be better measures of ration density than the data obtained by the original procedure, for they were highly reproducible and varied in a linear manner with the level of either the cellulose or kaolin in the diets. It is of interest to note that as the level of cellulose in the diets increased, the dry densities of the feeds decreased, whereas the densities measured by water displacement increased. Obviously the type of density values used would have a profound influence upon the interpretation of the experimental data. For the reasons stated previously and because feed becomes wet once it enters the crop of the chicken, the water displacement density values were used in the interpretation of the data resulting from this experiment.

The weight change and feed consumption data, though recorded on a per pen basis, were reduced to a per bird basis in order to simplify the handling of the data in subsequent statistical analyses. The statistical treatments were based on methods described by Snedecor ('56). To facilitate the interpretation of the results, data from diet groups 1 to 8 (zero to 42% of cellulose) and groups 1 plus 9 to 15, (zero to 42% of kaolin) were sometimes analyzed separately. When the observations from all treatments were pooled the data resulting from diet treatment 1 (100% basal) were employed only once.

RESULTS AND DISCUSSION

The weight gain and feed consumption data have been summarized and are presented in the form of means in table 3. The data show that the replacement of 6% of the basal diet with non-nutritive cellulose resulted in only a very small decrease in weight gain but that at a replacement of 12% or more the weight gain was considerably less than that of the birds fed the basal diet with no diluent. The birds receiving the diets diluted with kaolin consistently made greater weight gains than the birds receiving an equivalent level of the cellulose and showed no reduction in weight gain at a dilution of 12%. These data suggest that chicks are better able to deal with diets diluted with kaolin than with diets extended with the cellulose.

TABLE 3

Mean weight gain and feed consumption data expressed on a per bird basis

Treatment		Average weight[1]		Weight gain		Feed consumed		Feed consumed		Basal consumed		Protein consumed		ME consumed	
Basal	Diluent	C	K	C	K	C	K	C	K	C	K	C	K	C	K
%	%	*gm*		*gm*		*gm*		*ml*		*gm*		*gm*		*Cal.*	
100	—	294		100		224		158		224		53		661	
94	6	286	293	98	102	234	247	164	166	220	228	53	54	655	689
88	12	273	290	91	100	239	250	166	162	210	220	49	51	628	652
82	18	259	278	80	88	237	251	164	156	194	206	46	47	578	610
76	24	249	268	63	85	234	268	160	164	178	203	41	47	538	616
70	30	232	258	51	67	219	260	150	153	153	182	36	40	462	567
64	36	210	250	31	62	197	279	134	161	126	178	30	42	376	550
58	42	199	241	20	54	190	296	128	167	110	172	26	41	334	539

[1] C refers to diets diluted with non-nutritive cellulose (Alphacel), K to diets diluted with kaolin.

Analysis of variance supported this interpretation; the weight gains of the birds receiving diets 2 to 8 (6 to 42% of cellulose) being significantly less ($P < 0.01$) than those fed diets 9 to 15 (6 to 42% of kaolin).

The weight of feed consumed by the birds receiving the diets diluted with the cellulose increased as the level of diluent increased, up to a maximum of 12% dilution, and then decreased as the degree of dilution increased. These data suggest that the birds attempted to compensate for the reduced nutrient content of the diet by increasing feed intake. That the birds were unable to do this when the diets contained more than 6% of cellulose is demonstrated by the data pertaining to the basal diet, protein and ME consumption. The groups receiving the kaolin-diluted diets, with one exception, increased the weight of feed consumed as the level of diluent increased, again indicating an attempt to maintain nutrient intake. When the level of diluent in the diet exceeded 12% the intakes of basal diet, protein and ME decreased rapidly though not to the same degree as when cellulose was the diluent. It is apparent that the variations in the weight gain data, associated with the types and levels of diluents, are reflections of the variations in nutrient intake.

The data pertaining to the volume of feed consumed are of interest not only because they reflect the attempts of the birds to compensate for the reduced nutrient concentration of the diets by increased feed intake but also because they appear to be associated with the average body weights of the chicks. Regression analysis of the volume of feed consumed (Y) and the average chick weight (X) data yielded the highly significant ($P < 0.01$) correlation coefficient: $r = 0.687$ at 58 degrees of freedom. This indicates that the volume of feed consumed was largely regulated by the body weights of the birds. As the diluents appeared to inhibit weight gain in treatment groups 3 to 8 (12 to 42% of cellulose) and groups 11 to 15 (18 to 42% of kaolin) only, the analysis was repeated using the data from these treatment groups alone. The correlation coefficient ($r = 0.763$ at 42 d.f.) was larger than that obtained when all the data were employed (0.687), and again proved to be highly significant ($P < 0.01$). The regression equation resulting from this latter analysis was

$$Y = 42 + 0.4578X$$

where Y is the volume of feed consumed (ml) per bird per week, and X is the average weight per bird during the week. The relationship between the volume of feed consumed and body weight helps to explain why the birds receiving the kaolin-diluted diets made greater weight gains than those receiving diets containing the non-nutritive cellulose. Kaolin and cellulose had densities of 2.500 and 1.566 gm/ml respectively; consequently, a basal diet diluted with the cellulose would contain less nutrients per unit volume than when diluted with the same percentage by weight of kaolin. Therefore, when the volume of feed consumed is a limiting factor, birds receiving a kaolin-diluted feed would consume more nutrients than those receiving feed containing an equivalent proportion by weight of the cellulose. This difference in nutrient intake would influence the body weight gains of the birds.

The foregoing explanation tends to oversimplify the effects of ration diluents on the weight gains of growing chicks. The intake of nutrients from diluted feeds is probably reduced initially primarily because the birds are unable to consume a sufficient volume of diluted feed to compensate for its reduced nutrient concentration. However, such factors as palatability and alterations in the microflora of the digestive tract cannot be ruled out as factors which might contribute to this decrease in consumption. The difference in nutrient intake would result in a difference in the body weight gains of the treatment groups so that within a very short time the weights of the birds would be different. The difference in body weights would then result in greater differences in feed intake which would cause an even greater disparity in the weight gains. An attempt was made to measure the relative influences of basal diet consumption, average chick weight, ration density and basal diet concentration in the feed on the weight gains of the birds used in the present experiment. Multiple regression equations and correlation coeffi-

TABLE 4

A summary of simple correlation coefficients[1]

Variables		Correlation coefficients	
Y	X	0–42% cellulose[2]	0–42% kaolin
Weight gain	Basal diet consumed	0.969	0.957
	Average chick weight	0.948	0.930
	Ration density	−0.961	−0.911
	Basal diet, gm/ml of feed	0.986	0.945
Basal diet consumed	Average chick weight	0.981	0.943
	Ration density	−0.948	−0.879
	Basal diet, gm/ml of feed	0 959	0.902
Average chick weight	Ration density	−0.974	−0.904
	Basal diet, gm/ml of feed	0.980	0.931
Ration density	Basal diet, gm/ml of feed	−0.997	−0.961
Weight gain	M E Cal./gm of feed	0.967	0.929
	M E Cal./gm of feed	0.976	0.938

[1] Each correlation coefficient had 30 degrees of freedom.
[2] Alphacel.

cients were derived and comparisons were made between the partial regression coefficients as suggested by Snedecor ('56). These analyses proved to be of little value, for, as Snedecor ('56) explains, when a high correlation exists between any two of the X variables "even 6 to 8 significant figures may not be sufficient to control rounding errors." As will be seen from the data of table 4 the variables studied were highly correlated, one with the other.

As the volume of feed which may be consumed appears to be largely regulated by the body weight of the bird to which it is fed, it would seem that the concentration of nutrients within a feed may be of considerable practical importance. A regression analysis between the weight gain (Y) and ME per gram of feed (X) data yielded the highly significant ($P < 0.01$) correlation coefficients 0.967 and 0.929 at 30 d.f. for the diets diluted with cellulose (diets 1–8) and kaolin (diets 1, 9–15), respectively. The regression coefficients 71.20 and 45.84 resulting from this analysis were significantly different ($P < 0.01$). This indicates that the regression equations, while capable of predicting the weight gains of birds once the ME content of 1 gm of feed is known, are limited in use as they differ between types of diets. When the analysis was repeated using the data for weight gain (Y) and for the ME per milliliter of feed (X) the correlation coefficients again proved to be significant ($P < 0.01$): $r = 0.976$ and 0.938 at 30 d.f. for diets 1 to 8 and 1 plus 9 to 15, respectively. The regression coefficients 53.66 and 52.71 were not statistically different suggesting that the data for all diets might be pooled. Analysis of the pooled data yielded the significant ($P < 0.01$) r value of 0.966 at 58 d.f. and the regression equation

$$Y = -119.3 + 52.78X$$

where Y is the gain in weight per bird per week, and X is the ME per milliliter of feed. This demonstrates clearly that the concentration of nutrients in a diet measured in terms of units of nutrient per milliliter is a more useful statistic than concentration measured as units of nutrient per gram of feed. The equation relating to weight gain and ME concentration has been used solely to demonstrate the importance of measuring nutrient density. In the present experiment the ratios of nutrients, one to the other, remained relatively constant as dilution was at the expense of the whole diet; consequently, one would expect that similar results would have been obtained had the concentration of another nutrient been studied.

The influence of nutrient density on weight gain might help to explain such phenomena as the extra-caloric effects of fat (Hill et al., '58) and the improvement of feed utilization by pelleting (Allred et al., '57a, '57b).

SUMMARY

A chick starter diet diluted, by total ration replacement, with zero, 6, 12, 18, 24, 30, 36 and 42% of cellulose and kaolin was fed ad libitum to growing chicks for a three-day ration adustment period followed by a 7-day experimental period. During the latter period, weight gain and feed consumption data were recorded. The 15 diets were each assigned to 4 replicated pens of 14 birds.

The densities of the diets were measured by both a "conventional dry procedure" and by water displacement; the latter proved to be a more satisfactory measure.

The birds receiving the diluted diets attempted to compensate for reduced nutrient concentration by increased feed intake; however, except for the groups receiving diets containing 6% of cellulose and 6 and 12% of kaolin the attempts were unsuccessful. Reduced nutrient intake resulted in decreased weight gains which further limited nutrient intake as this latter variable was largely associated with body weight.

A linear relationship between the concentration of nutrients per unit volume of feed and the weight gains of the chicks suggested that maximum weight gains may be obtained only with diets of high nutrient concentration. Nutrient concentration expressed in terms of nutrients per unit weight did not prove to be as satisfactory a measure as one which took into account ration density.

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EXPERIMENTAL STUDIES OF THE EFFECT OF KAOLIN-SiO_2 MIXTURES ON THE ORGANISM. (Etudes Experimentales De l'action du caolin et melanges de caolin et de sio sur l'organisme)

By

Rodica Tripsa

of

Romanian Institute of Hygiene and Public Health, Division of Occupational Health, Bucharest

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EXPERIMENTAL STUDIES OF THE EFFECT OF KAOLIN AND KAOLIN-SiO_2 MIXTURES ON THE ORGANISM

By

Rodica Tripsa

The studies by the Canadian authors* as well as the investigations of the Saranak investigators have shown that certain minerals containing the Al, Mg and Fe ions reduce the solubility of SiO_2. In vivo experiments have demonstrated a considerable reduction in the progress of silicosis in animals that have been subjected to inhalation of SiO_2 together with metal aluminum dust or aluminum hydroxide dust. Silicates are combinations of silicon with metals, especially aluminum. Their stability is similar to that of crystalline silicon dioxide. In vitro experiments carried out by KG and MG** have shown that kaolin dust reduces the solubility of SiO_2.

The solubility of SiO_2 in mixtures of SiO_2 and kaolin is summarized in Table 1. Natural silicates release very few silicone ions as indicated by the data in the table for 2 mg of dust suspended in 100 ml of ascites fluid (SiO_2 in mg per 100 of ascites fluid).

Therefore, kaolin releases a very small amount of Si ions and simultaneosly, lowers the solubility of SiO_2

The kaolin used during the experiments described in the present report was obtained from a very pure deposit found at Horni Briza (Czechoslovakia) and contains very little SiO_2. Its composition is shown below:

Kaolin.....	92.3%
Muscovite.....	4.3%
Feldspar.....	1.4%
SiO_2.....	1.4%

* Denny, Robson & Irvin
** King & Mac Georges

The dusts were obtained by elutriation with distilled water, their particle size being of the order of 0.5 to 3 microns, with the largest fraction fluctuating around 1.5 μ.

The silicon dioxide used was 97% pure and was obtained by the milling of pure sand. Its particle size varied between 0.8 and 4 μ.

In order to obtain an accurate picture of the chronic effects of pure kaolin and of the effects of the kaolin-SiO_2 mixtures, we used intratacheal instillation, intravenous injection and free dust inhalation in an exposure chamber. Peritoneal injection was used only in a small group of animals treated with pure kaolin. The experiments were carried out in age-matched rats weighing 160-190 g. Intratracheal instillation was used to establish both the effect on kaolin on the course of silicosis and the sensitivity of the method for evaluating the activity of the dust mixtures.

Table 1

	First day	Third day	Sixth day
2 g SiO_2	4.4	5.4	7
2 g kaolin	0.1	0.1	0.1
2 g SiO_2 + 2% kaolin	2.7	4.8	4.5
2 g SiO_2 + 4% kaolin	1.8	2.3	2.4
2 g SiO_2 + Al_2O_3	0.4	0.3	0.3

As controls for the tracheal instillation experiments we used a series of animals instilled with impure crystalline SiO_2 (5-80 mg). The results of the experiments obtained with a dose of 5 mg were not taken into consideration since this particular amount failed to produce a fibrotic reaction. The effect obtained with 80 mg was also not taken into consideration since the large scope of the lesions induced by this particular quantity could result in the appearance of artifacts which, in turn might obstruct any cumulative or inhibitory effect.

Table 2

Agent	First day	Third day	Sixth day
Quartz	4.4	5.4	7
Sericite	-	1.7	1.8
Chlorite (mass)	-	1.1	1.4
Chloride (sax)	-	1.0	1.2
Kaolin	0.1	0.1	0.1
Biotite	-	-	1.1

Accordingly, for control purposes we used only the data obtained with SiO_2 in doses of 10, 20 and 40 mg.

Intratracheal instillation was applied to 4 lots of animals treated according to the following schedule:

first lot: 10 mg kaolin + 30 mg SiO_2

second lot: 20 mg Kaolin + 20 mg SiO_2

third lot: 30 mg kaolin + 10 mg SiO_2

fourth lot: 40 mg kaolin

In this manner, the total dose of 40 mg kaolin was achieved with various concentrations of SiO_2. Each experimental series consisted of 14 to 19 animals. Groups of animals were sacrificed at 4, 7, 9 and 11 months.

The histological features of the first three lots are highly similar to each other, especially during the first 3-5 months. During this particular period, the lung changes are characterized by the presence of alveolitis of average severity, similar to that induced by 40 mg of pure SiO_2. The cellular nodiles appear sometimes between the fifth and seventh month. Certain differences between the various lots of animals are evident during the more advanced stages (after 9 and 11 months), during the period of collagenous metaplasia of the nodules. However, these differences are smaller than those

induced by doses containing SiO_2

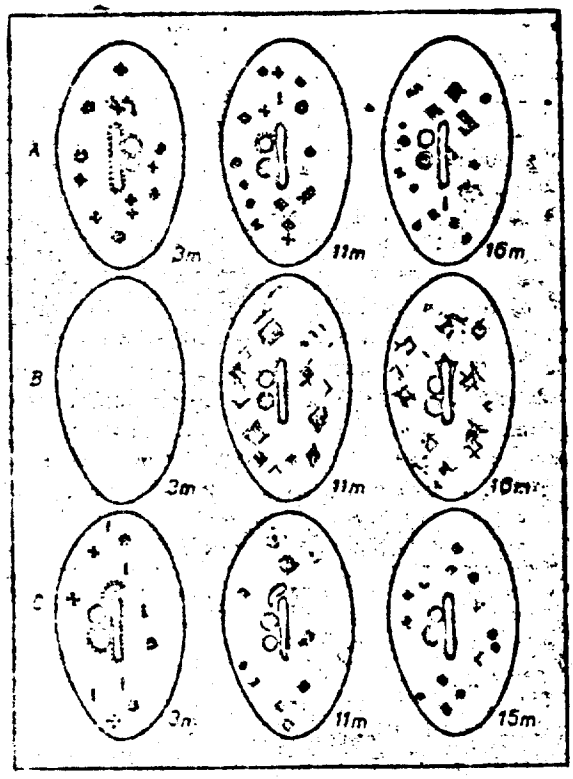

Fig. 1. Cumulative effect of kaolin and of montmorillonite administered by tracheal instillation. - A) Instillation of 30 mg kaolin + 10 mg SiO_2, 11 months. Conglomerated fibrocellular nodules (Van Gieson 6 × 10). B) 11 months following the instillation of 30 mg montmorillonite + 10 mg SiO_2. Highly advanced emphysema. Fibrocellular nodules.(4 × 10, hematoxylin-eosin). - C) 11 months following the instillation of 10 mg SiO_2. Cellular nodules with a fine reticulum of collagenous fibers (Van Gieson, 6 × 10).

All the kaolin-containing mixtures induce a more advanced degree of silicosis than would the amount of SiO_2 present in the mixture. Fibrosis appears earlier than in the respective lots treated with pure SiO_2. Furthermore, the degree of peribronchial and perivascular fibrosis is more severe than that induced by the respective doses of pure SiO_2. In certain cases, the changes observed were not more severe than those induced by SiO_2 alone, but there were also cases where the extent of the response was more advanced. As a result of these individual variations and due to the relatively uniform

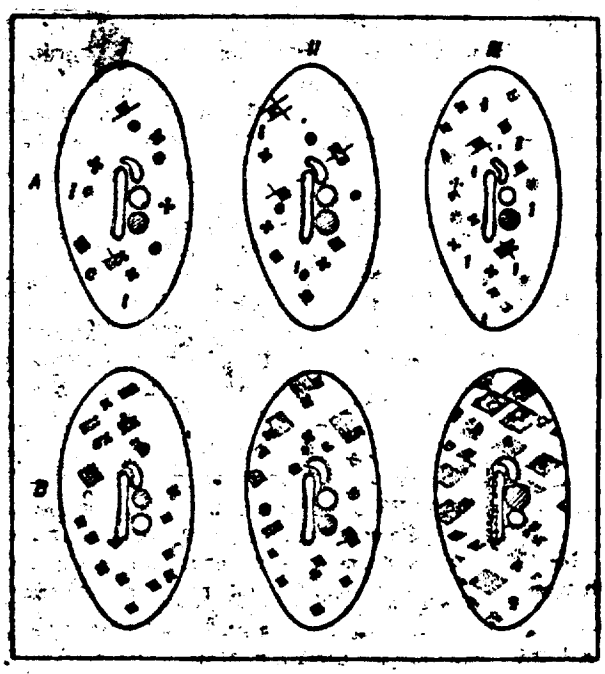

Fig. 2. - Differential evolution of silicotic lesions following the inhalation of pure SiO_2 and the same amount of SiO_2 mixed with threefold higher dose of kaolin. I - 9 months; II - 9 month inhalation, 15 month disease; III - 15 month inhalation. - A) SiO_2, 7000-10000 particles/cc. - B) SiO_2, 7000-10000 particles/cc + kaolin, 30000 particles/cc.

changes, the lots where the difference in SiO_2 was of the order of 10-20 mg could not be defined accurately. The most significant cumulative reaction was observed in the lot receiving 10 mg SiO_2 + 30 mg kaolin, as compared to the mixture containing 10 mg kaolin.

After 9 to 11 months, SiO_2 induces the appearance of small and sparce peribronchial nodules without a collagenous reticulum between the dust cells, as well as the onset of vascular or bronchial fibrosis. At the end of the same time period, in certain cases, the administration of 10 mg SiO_2 + 30 mg kaolin induces the formation of highly fibrotic pseudotumoral masses of significant size. Examination of animals that survived 15 months confirms the development of these masses.

Kaolin alone induced a strong proliferative effect. In animals sacrificed during the earlier stages, the reaction of the lung macrophages was similar to

that induced by dust containing SiO_2. Significant alveolitis is also present during the first 3 months. At 3 to 7 months, small cellular nodules are formed, similar in certain respects to the nodules induced by SiO_2 during the earlier stages, the only difference in silicosis being the presence of young cells and the slight infiltration of the intraalveolar septa. At 7 months, instead of being enlarged and merging together, the nodules display involutional characteristics while the lung infiltration legions clearing to the point that at 11 months, the lung has almost an intact appearance. The only trace of the intensive cellular circulation occurring during the earlier stages is a slightly more evident ring of perivascular connective tissue.

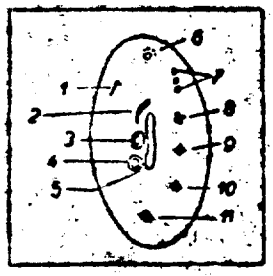

Fig. 3. - 1) Interstitial fibrosis; 2) vein; 3) lymphatic nodule; 5) bronchus; 6) periarterial infiltration with dust cells; 7) alveolix with few cells; 8) alveoli full of dusty cells; 9) cellular nodules with a fine mesh; 10) fibrocellular nodule; 11) hyaline nodule

Montmorillonite is a variety of kaolin having a colloidal structure groups of rats were treated with fractioned doses of montmorillonite obtained from two different deposits, for a total dose of 40 mg of pure montmorillonite. One more group of animals was treated with 30 mg montmorillonite and 10 mg SiO_2. All animals treated with 40 mg montmorillonite failed to survive and their lungs displayed significant acute lesions. At 11-12 months after instillation, the animals treated with 30 mg montmorillonite and 10 mg SiO_2

manifested extensive lung fibrosis, significant emphysema and a relatively large number of cellular nodules. The general appearance of the alterations was different from that of other forms of pneumoconiosis. Thus, emphysema was the main lesion, fibrosis starting from the alveolar walls altered by emphysema and not from the nodules, as is the case in silicosis. The pulmonary lesions preceded the ganglionic lesions which, due to the moderate amount (40 mg) of dust used, is not the case for any other type of pneumoconiosis induced by SiO_2 or by mixtures. All these features lead us to consider that one portion of the lesions represents a consequence of toxic intersittial pneumonia subsequent to the acute effect of montmorillonite. However, the cellular nodules with their collagen-containing reticulum are much larger than those produced by treatment with 10 mg SiO_2, and the alterations of the tracheobronchial ganglia (a consequence of slow cell migration) are greater than those yielded by pure SiO_2. All these changes appear gradually and cannot be the result of the acute effects of montmorillonite (fig. 1).

The unexpected cumulative results obtained with the two varieties of kaolin prompted us to investigate the effect of kaolin administered by means of intravenous injection.

The intravenous injection method has been used by G* et al. to test the inhibitory activity of aluminum hydride. The results obtained by these investigators are similar to those obtained during the inhalation experiments. The same method was also used by K** et al. to determine quantitively the amount of collagen present in silicotic lesions by means of chemical analysis.

We used the following quantitative criteria: number of nodules and their average size; area occupied by the silicotic granulomas in the liver and in the spleen with respect to the normal tissue of these organs. The counting and size determination was carried out in a series of 100 microscopic fields of the same

* Gardner

** King

size. As was the case with intratracheal instillation, in each case the stage of the fibrotic lesions was determined by examining the thickening and increase in the density of the reticulin reticulum according to the degree of argentaphinic impregnation. The appearance of the collagen fibers and their gradual evolution into a massive hyaline form was performed by means of the Van Gieson and Trichrome Masson staining techniques.

The relatively significant acute toxicity induced by kaolin showed that the total dose of kaolin does not exceed 6.8 mg per injection. The experiments were carried out in two groups of animals, one group receiving 20 mg kaolin and 20 mg SiO_2 and the other 40 mg kaolin only.

The control groups were treated with gradually increasing doses of SiO_2. In these animals, the differences between the alterations caused by SiO_2 doses of 10, 20, and 40 mg were small but significant enough to perform evaluation regarding any eventual inhibitory or exacerbating activity. Despite the highly detailed criteria applied, the data regarding the measurement of lesion size, the ratio between the area occupied by the silicotic granulomas and that of the healthy tissue, the differences between the changes induced by the various doses are less significant than those obtained subsequent to intratracheal instillation. Nevertheless, the conclusions from the intravenous injection experiments are in agreement with those drawn from the instillation experiments.

Kaolin exhibits a definite cumulative effect. The lesions evolve gradually and their area is similar to that obtained at 12 months with 40 mg of SiO_2. At the end of 18 months, the fine collagen network observed in the 12th month becomes more evident, especially in the perivascular region. At this stage, it resembles that found at the end of 18 months in animals treated with

40 mg SiO_2. The hypertrophic ganglion contains significant numbers of dust cells with a thick network of collagen fibers. The same was true for animals treated with 10 mg SiO_2 + 10 mg kaolin.

The injection of kaolin in gradually increasing doses of 10, 20 and 40 mg failed to produce gradually evolving lesions of the silicotic nodule type in the spleen and in the liver of the animals. Animals examined at 18 months following intravenous injection displayed only a few isolated groups of cells in some Kiernan spaces, without trends towards gradual fibrosis. The area occupied by these cells is minimal compared to that occupied by the healthy tissue. At 6 to 18 months after treatment, the time evolution is practically non existent and the quantitative differences between the various experimental doses are small (amounting only to a few percentage points of the entire area studied).

It is quite evident that the results of the experiments carried out with the two methods are similar with respect to the effect of pure kaolin. These results seem to be in disagreement with those obtained by certain other investigators (R)* who studied the action of pure Kaolin administered intraperitoneally. These authors drew the conclusion that kaolin has fibrogenic activity similar to that of SiO_2.

We also used the intraperitoneal technique to inject pure kaolin (50 mg) in experimental animals, while a control group received 50 mg of crystalline SiO_2 in the same dispersion mentioned earlier. Two months following the intraperitoneal administration of kaolin, the animals displayed nodules similar to those induced by pure SiO_2 in the epiploon, in the mesenterion and in the intestinal ansae. Histological examination revealed a rather significant capsule of connective tissue, a phenomenon similar to that produced by silicon dioxide. However, the course of the phenomenon was manifested at a slower rate. The

* Ruttner

mesenteric ganglia failed to develop the fibrotic lesions characteristic of dusts containing SiO_2 even 12 month or more after treatment. Furthermore, these ganglia exhibited little hypertrophy while a few clusters of dust cells representative of cell migration, with no other signs of evolution appeared only after 12 months.

As for the case of pure silicates, these experiments demonstrate once more the relative value of the results obtained with the peritoneal injection method.

As for the effect of kaolin on the evolution of silicosis, our experiments performed with both methods show that it has a cumulative action.

Data obtained from the literature (G, S, K)* show that inhalation of dust mixtures produces benig n penumoconiosis progressing at a very slow rate, practically compatible with the normal life of the experimental animal. Following the inhalation for 3-4 years of granite dust containing 30% SiO_2, G obtained only the occurrence of isolated silicotic nodules. Furthermore, during a 2-year period, the reaction was mainly of an alveolar nature.

At the same time, the cumulative effect of inhaled kaolin was not known.

We subjected two groups of animals to simultaneous inhalation of the same amount of quartz dust. The second lot also received simultaneously a dose of kaolin almost three times higher. Thus, one inhalation chamber was set for 7-10, 000 SiO_2 particles per cc of air while another chamber was set for 7-10,000 SiO_2 particles + 40-50,000 kaolin particles. The concentration of dust in the air was acheived according to the model described by C** (a Soviet investigator, with a few modifications. Due to the fact that the supply of SiO_2 dust was regulated by a single distributor, any variation in the concentration of SiO_2 in the air in one chamber was also reflected in the other.

* Garner, Schepers, King
** Chakbazian

Accordingly, the animals in both chambers received the same amount of SiO_2 dust (Fig. 2).

As for the previous tests, the animals used for the inhalation experiments were white rats. The animals were kept in the dusty atmosphere for 6 hours and sacrificed 7, 30 and 60 days, and at 4, 6, 9, 15 and 22 months. Histological examination of the lungs and mediastinal ganglia obtained from the animals sacrificed was carried out according to the same criteria as in the preceding experiments. In animals sacrificed during the first few months of the experiments, alveolitis had the same features as during the instillation experiments, that is a radical change in the lungs. Although the dust concentration in the inhalation chamber containing the mixed dust was 4 times greater and although alveolitis is not a dust-specific reaction, the degree of alveolitis induced by the mixture of silicon dioxide and kaolin was no greater than that obtained in the animals inhaling pure silicon dioxide.

Animals sacrificed at the end of 4 months displayed the first signs indicative of the future formation of silicotic nodules. In certain areas, the intraalveolar septae were thickened and there was an accumulation of dusty cells filling the alveoli in this zone. There were many phagocytes with a globular appearance and a clear protoplasm which, at times, had a small nucleus. The septa were moderately infiltrated by dust cells having an aged appearance and containing a tachychromatic nucleus, but little protoplasm. At this stage also there were no significant differences between the two lots of animals.

Animal sacrificed at the end of 9 months displayed characteristic perivascular nodules and even nodules independent of the vessels and of the bronchi. There was a relatively sparse reticulin reticulum which at times exhibited collagenous metaplasia close to the alveolar walls, surrounded by the cell conglomerates.

The hypertrophic nodules contained large conglomerates of dusty cells in a reticulin reticulum. There were no significant differences between the group of animals treated with quartz and those treated with pure quartz, both with regard to ganglionic hypertrophy and the reticulin reticulum. The main difference between the two groups was the extent of the lesions which proved to be slightly fewer than those induced by pure SiO_2. However, this difference was not significant.

Starting at this particular stage, we removed some of the rats belonging to the two lots from the inhalation chambers and let them inhale pure air. Accordingly, during the next stage (that is, at 15 months from the onset of the experiment) we compared 4 lots of animals, including two lots (with and without kaolin) that inhaled dust for only 9 months, although the duration of their condition totalled 15 months.

At the end of 15 months, the differences between the two kaolin and SiO_2 lots were highly significant as compared to those treated with pure SiO_2. The lesions were 3-4 times greater and this trend increased in the lot of animals subjected to continuous dust inhalation for 15 months. In addition to large nodules arranged crown-like and occupying a large area at the periphery of the lung, observed in both lots treated with kaolin, the nodules of animals subjected to continuious dust inhalation for 15 months were connected by zones of extensive alveolitis.

In the peripheral zone the reticulum of the nodules exhibited definite collagenous fibers. In addition, some of them also exhibited collagenous metaplasia in the central star. The bronchial changes were much more extensive than in the animals treated with pure SiO_2. As a result of fibrosis, the internal diameter of the bronchus became narrower and the alasticity of the bronchus declined. Furthermore, the ganglia were larger and more fibrotic than in cases caused by the inhalation of pure silicon dioxide.

The area occupied by the nodules and by the evolving alveolitis represented more than 50% of the area of the lung sections, as compared to 10-15% occupied by the nodules observed in the lot of animals treated with pure SiO_2. In the control rats, the nodules were scarcer and smaller, although they also contained a reticulum rich in collagen fibers.

Dust inhalation was continued for 18 months and the last animals were sacrificed at 22 months following the initiation of the experiments. At this particular time, none of the animals treated with the mixture of kaolin and SiO_2 was alive, (all died as a result of very extensive lesions). Animals subjected to the inhalation of pure silicon dioxide displayed a pseudotumoral silicosis with almost fully hyalinized nodules occupying a large area of each lobe and highly advanced fibrosis. The bronchi displayed atrophic changes and significant perivascular fibrosis which affected the walls of the vessels.

Conclusions

Regardless of the experimental method used, kaolin induced an enlargement of the silicotic lesions. The degree of this enlargement was proportional to the amount administered and the stage (time from the onset) of the experiment. Kaolin alone, regardless whether it was administered by intratracheal instillation or intravenous injection, induced minimal changes, without progressive tendencies (increase or fibrous metaplasia).

The results obtained subsequent to intraperitoneal injection do not agree with the other experiments, being also refuted by the benign evolution of the ganglionic changes in all lots, even in those where intraperitoneal introduction was carried out. The results obtained with all the other methods described in the present study were in agreement with each other.

The clinical data confirm the fact that kaolin has a cumulative effect, especially subsequent to the inhalation of large amounts.

L*and McIver give details on the lung changes in workers with several years exposure to kaolin dusts. These investigators examined the lungs of 2 workers employed in the extraction and preparation of kaolin, who died as a result of pneumoconiosis. The lungs and ganglia of one of these men, with a 17-year occupational history, presented significant increase of the interalveolar peribronchial tissue as well as pleural thickening.

The other case (a 35 year old individual who worked for 21 years in the preparation of kaolin) displayed massive areas of lung fibrosis, vascular deformation and pleura thickening. The lymphatic ganglia exhibited large areas of fibrotic collagen. Hale, Gough, King & Nagelschmidt reported clinical and rediological findings in 6 advanced cases of kaolinosis in Cornwall. The autopsy findings in one of these cases were compared with those obtained in another such cases reported in Georgia (U.S.A.). In both instances the postmortem findings revealed very large amounts of kaolin (20-40 g) in the lungs, together with a small concentration of crystalline SiO_2.

Summary

The effect of certain clay silicates, kaolin and montmorillonite on the development of silicosis was investigated in several groups of white rats.

Histological changes occurring in the lungs, liver, spleen, peritoneum and lymphatic nodules of the rats, at 11 to 15 months following intratracheal instillation, intravenous and intraperitoneal injection and dust inhalation, are described.

Intratracheal instillation or intravenous injection of pure kaolin failed

* Lynch

to produce lesions. Peritoneal administration of pure kaolin resulted in the formation of fibrotic nodules while the lymphatic nodes were not affected.

Mixtures of SiO_2 and kaolin, in various proportions, demonstrated the cumulajtive action of kaolin, regardless of the route of administration.

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ETUDES EXPERIMENTALES DE L'ACTION DU CAOLIN ET MELANGES DE CAOLIN ET DE SiO_2 SUR L'ORGANISME

RODICA TRIPŞA

L'Institut d'Hygiène et de la Santé Publique de Roumanie, Section d'Hygiène du Travail, Bucureşti

Les études des auteurs canadiens Denny, Robson, Irvin, comme aussi celles de Gardner et ses collaborateurs de Saranak ont montré que certains minéraux contenant des ions métalliques — Al, Mg, Fe — réduisent la solubilité de la SiO_2. Les expériences *in vivo* ont démontré un ralentissement sensible de l'évolution de la silicose chez les animaux qui ont inhalé simultanément de la SiO_2 et des poussières d'aluminium métalique ou d'hydrate d'aluminium. Les silicates sont des combinaisons du silicium avec des métaux, surtout avec l'aluminium, aussi stables que le bioxyde de silicium cristallin. Les expériences in vitro de King et Mac Georges ont montré que la poussière de caolin réduit la solubilité de la SiO_2.

La solubilité de la SiO_2 dans les mélanges de la SiO_2 avec du caolin en mg. de la SiO_2 (Tab. 1.) Les silicates naturels libèrent très peu d'ions du silicium, comme il apparait du tableau 2 g de poussière en suspension dans 100 ml de liquide d'ascite de la SiO_2 en mg. pour 100 ml de liquide ascitique.

Le caolin libère donc une très petite quantité d'ions du Si et en même temps abaisse la solubilité de la SiO_2.

Le caolin utilisé dans nos expériences, provenait d'un gisement très pur de Horni Briza (Tchécoslovaquie) et ne contient que très peu de la SiO_2:

92,3% Caolin
4,3% Muscovite
1,4% Feldspathe
1,4% SiO_2

Les poussières ont été sélectionnées par élutriation dans l'eau distillée, leur dispersion étant de l'ordre de 0,5 à 3 microns, la majorité se trouvant toutefois aux environs de 1,5 microns.

Le bioxyde de silicium utilisé avait une pureté de 97% et provenait du moulinage de sable pur, ayant une dispersion entre 0,8 et 4 microns.

Afin d'obtenir une image plus complète de l'effet chronique du caolin pur et des mélanges de caolin et de la SiO_2 nous avons utilisé les methodes des

instillations intratrachéales, des injections intra-veineuses et de l'empoussiérage libre dans une cabine. L'injection peritoneale n'a été utilisée que chez un petit lot des animaux qui ont reçus du caolin pur. Nous avons utilisé des rats blancs du même âge, avec le poids entre 160—190 grames. Les instillations intratrachéales ont eu pour but d'établir tant l'action du caolin sur l'évolution de la

Tab. 1.

	Premier jour	Troisième jour	Sixième jour
2 g SiO_2	4,4	5,4	7
2 g caolin	0,1	0,1	0,1
2 g SiO_2 + 2% caolin	2,7	4,6	4,5
2 g SiO_2 + 4% caolin	1,8	2,3	2,4
2 g SiO_2 + 1% Al_2O_3	0,4	0,3	0,3

silicose que la sensibilité de la méthode pour estimer l'action des mélanges de poussières.

Dans l'instillation trachéale nous avons utilisait pour témoins une série des animaux nistillés avec de la SiC_2 cristalline non pure (entre 5—80 mg). On a éliminé la dose de 5 mg parce qu'elle ne produisait pas une réaction fibreuse e aussi celle de 80 mg parce que la grande étendue des lésions eût pu provoque des artefacts qui, à son tour, aurient pu masquer l'effet cumulatif ou inhibiteur

Tab. 2.

Substance	Premier jour	Troisième jour	Sixième jour
Quartz	4,4	5,4	7
Séricite	—	1,7	1,6
Chlorite (mass)	—	1,1	1,4
Chlorite (sax)	—	1,0	1,2
Caolin	0,1	0,1	0,1
Biotite	—	—	1,1

Nous avons utilisé donc, comme lots témoins dans nos expériences, les doses de 10, 20 et 40 mg de SiO_2.

Par l'instillation intratrachéale nous avons fait des expériences sur 4 lots recevant:

le premier lot: 10 mg caolin + 30 mg de SiO_2,
le deuxième lot: 20 mg caolin + 20 mg de SiO_2,
le troisième lot: 30 mg caolin + 10 mg de SiO_2,
le quatrième lot: 40 mg caolin.

On réalise de cette manière la dose totale de 40 mg avec des concentrations variables de la SiO_2. Chaque lot était constitué de 14 à 19 rats. Leur sacrification a été effectuée par groupes après 4, 7, 9 et 11 mois.

L'aspect histologique des premiers trois lots est très ressemblant, surtout pendant les premiers 3—5 mois. Pendant cette période les modifications pulmonaires sont caractérisées par une alvéolite de moyenne étendue, semblable à celle produite par 40 mg de la SiO_2 pure. C'est entre le 5 et 7 mois que des nodules cellulaires se sont formés. Dans les phases plus avancées, après 9 et

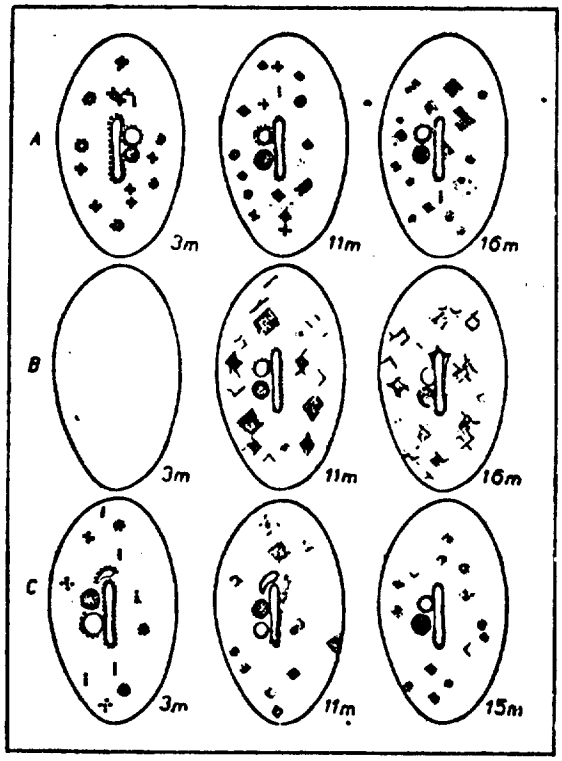

Fig. 1. L'action cumulative de caolin et de montmorillonite mise en évidence par l'instillation intratrachéale. — A. L'instillation de 30 mg de caolin + 10 mg de SiO_2, 11 mois. Nodules fibrocellulaires conglomérés (Van Gieson 6×10). B. 11 mois après l'instillation de 30 mg de montmorrillonite + 10 mg SiO_2. Emphysème très prononcé. Des nodules fibrocellulaires. (4 × 10 hematoxiline eosine). — C. L'instillation de 10 mg de SiO_2 — 11 mois. Nodules cellulaires avec un réticulum fin de fibres collagènes (Van Gieson 6 × 10).

11 mois, dans la période de la métaplasie collagène des nodules, on observe quelques différences entre les lots, pourtant ces différences sont moindres que celles provoquées par les doses contenant de la SiO_2.

Tous les mélanges avec caolin provoquent une silicose plus avancée que celle obtenue par la dose de la SiO_2 incluse dans le mélange. L'apparition de la fibrose précède celle chez les lots respectifs recevant de la SiO_2 pure. La

fibrose péribronchique et périvasculaire est plus accentuée que celle provoquée par les doses respectives de la SiO_2 pure. Les variations individuelles étaient assez importantes. Il y avait des cas, où les modifications n'était pas plus importantes que celles provoquées par la SiO_2 incluse dans les lots, mais il y avait aussi assez de cas où la réaction était plus importante. A cause de ces varia-

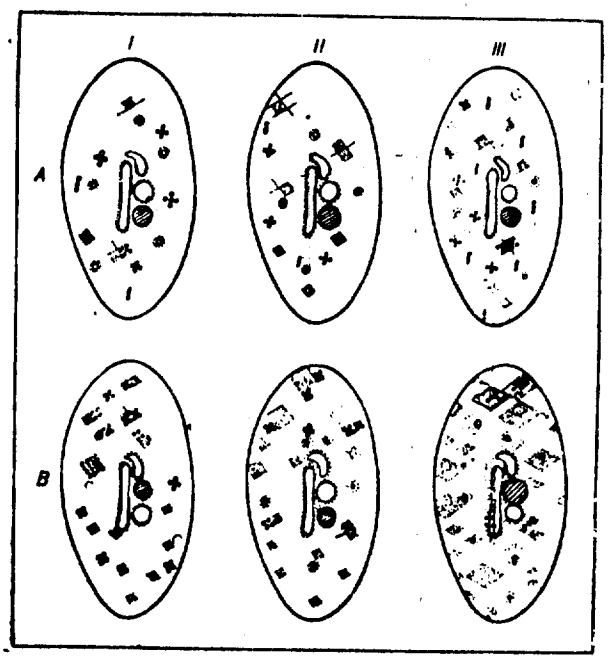

Fig. 2. Evolution différentielle des lesions silicotiques après l'empoussièrage à SiO_2 pure et avec SiO_2 dans la même quantité avec une dose trois fois plus forte de caolin. I 9 mois, II 9 mois empoussièrage, 15 mois maladie, III 15 mois empoussièrage. — A. SiO_2 7000 — 10000 part./cm³ — B. SiO_2 7000 — 10000 part./cm³ + caolin 30000 part./cm³.

tions individuelles et à cause des modifications relativement uniformes on ne peut pas individualiser, avec une très grande précision, les lots pour lesquels la différence de la SiO_2 était de l'ordre de 10 à 20 mg. La réaction cumulative la plus importante était observée dans le lot recevant 10 mg SiO_2 + 30 mg caolin en comparaison avec la dose chargée de 10 mg du caolin.

C'est après 9 jusqu'à 11 mois, que la SiO_2 détermine l'apparition de petits nodules perivasculaires et péribronchiques rares, pas commassés avec un réticule de collagène parmi les cellulles à poussière, avec un commencement de la fibrose du vaisseau ou de bronches. Après le même intervalle de temps les doses de 10 mg de la SiO_2 administrées ensamble avec 30 mg du caolin provoquent, dans quelques cas, la formation des masses pseudotumorales intensément fibrosées de dimensions importantes. L'examen des cas ayant survécus 15 mois a confirmé ce développement.

Le caolin pur lui-même a provoqué une vive action proliférative. Dans les premières étapes des sacrifications la réaction des macrophages pulmonaires était semblable à celle des poussières contenant de la SiO_2. L'alvéolite est aussi importante pendant les premiers 3 mois; entre 3 et 7 mois des nodules cellulaires petits se sont formés en quelques regards semblables aux nodules provoqués par la SiO_2 dans des phases moins avancées, C'est seulement l'aspect jeune de cellules et la faible infiltration des septes interalvéolaires qui la différencient de la silicose. Après 7 mois, au lieu de l'agrandissement et de la confluence de ces nodules, leur involution commence, le poumon se desinfiltre progressivement et vers 11 mois il devient presque intégral. Comme vestige de l'intense circulation cellulaire des étapes antérieures on peut observer seulement un anneau un peu plus accentué du tissu conjonctif périvasculaire.

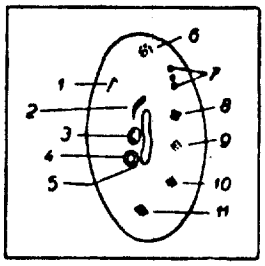

Fig. 3. Scheme: 1. fibrose interstitielle, 2. veine, 3. nodule lymphatique, 4. artère, 5. bronche, 6. infiltration periarterielle avec les cellules de poussière, 7. cellules rares dans les alveoles, 8. alveoles remplies de cellules de poussière, 9. nodule cellulaire avec une trame fine, 10. nodule fibrocellulaire, 11. nodule hyalin.

Le montmorillonite est une variété du caolin avec une structure colloidale. A deux groupes de rats on a été instillé du montmorillonite provenant de deux gisements différents, en de doses fragmentées, afin de totaliser 40 mg de montmorillonite pur et, dans une autre groupe, 30 mg de montmorillonite et 10 mg de SiO_2. Pas un seul animal du lot recevant 40 mg de montmorillonite n'a pas survécu, et à l'examen du poumon on a observé des lésions aigues importantes. Les animaux auxquels on a instillé 30 mg de montmorillonite et 10 mg de SiO_2 présent 11 à 12 mois après l'instillation une fibrose pulmonaire étendue, un emphysème accentué outre un nombre suffisamment élevé de nodules cellulaires. L'aspect diffère des autres pneumonioses, l'emphysème est la lésion essentielle, la fibrose a pour point de départ les parois alvéolaires modifiés par l'emphysème, et non pas les nodules, comme dans la silicose, les lesions pulmonaires précèdent celles ganglionaires ce qui n'arrive pas, à cause de la quantité modérée de poussières introduites (40 mg), chez aucune des pneumoconioses provoquées par la SiO_2 ou des mélanges. Tout cela nous oblige de considérer une partie des lésions comme la séquelle d'une pneumonie interstitielle toxique sous l'action aigüe du montmorillonite. Pourtant, les nodules cellulaires avec un réticulum marqué de collagène sont beaucoup plus grands que ceux provoqués par 10 mg de SiO_2 et les modifications des ganglions trachéobronchiques —

conséquence de la migration lente les cellules — sont plus grandes que celles provoquées par la SiO_2 pure. Ces changements se forment graduellement ne pouvant pas être la conséquence de l'action aigue du montmorillonite (fig. 1).

Les résultats cumulatifs inattendus des deux variétés de caolin nous les ont déterminé à expérimenter l'action du caolin par la méthode des injections intra-veineuses aussi.

Cette méthode a été utilisée par Gardner et ses collaborateurs dans le but de tester des actions inhibitrices du hydrate d'aluminium. Leurs résultats ont été similaires avec ceux obtenus par empoussièrage. La méthode a servi à King et ses collab. dans leurs études ayant pour but de déterminer la présence quantitative du collagène dans des lésions silicotiques par dosage chimique.

Nos critères pour l'appréciation quantitative ont été les suivants: nombre des nodules, leur grandeur moyenne, la surface occupée par les granulomes silicotiques du foie et de la rate en rapport avec le tissu sain des mêmes. La dénombration et l'appréciation des dimensions ont été éffectués sur 100 champs microscopiques de la même grandeur. Comme c'était le cas aussi chez les instillations intratrachéales, dans chaque cas on a comparé le degré d'évolution des lésions vers la fibrose, en examinant l'épaisissement et l'augmentation de la densité du réticulum de réticuline à l'aide de l'impregnation argentaphine et l'apparition des fibres collagènes et leur évolution vers le hyaline massif à l'aide des colorations Van Gieson et Trichrome Masson.

La toxicité aigue assez importante du caolin a montré que la dose totale du caolin ne dépasse pas 6,6 mg par injection. On a travaillé sur deux lots, le premier recevant 20 mg de caolin et 20 mg de SiO_2, et le deuxième 40 mg de caolin.

Les lots témoins, à qui l'on a administré des doses progressives de la SiO_2, ont montré des petites différences entre les doses de 10, 20 et 40 mg de SiO_2, mais suffisantes pour pouvoir apprécier une action inhibitrice ou exacerbante éventuelle. En dépit des critères très détaillés utilisés, les données concernant la mesure des dimensions des lésions, le rapport entre la surface occupée par les granulomes silicotiques et celle occupée par le tissu normal et la sensibilité de différentiation des doses sont moins importantes que celles obtenues dans les instillations. Les conclusions concordent, tout de même, avec les premières expériences.

L'action du caolin est nettement cumulative. Les lésions sont évolutives occupant une surface similaire à celle de 40 mg de SiO_2 à 12 mois. La trame fie de collagène dans le 12-ème mois s'accentue surtout périvasculairement vers le 18-ème mois. Aussi est-il similaire à celle de 18 mois de 40 mg de SiO_2. Le ganglion hypertrofié contient des masses importantes de cellulles de poussière avec une trame dense de fibres collagènes. Une dose de 10 mg de SiO_2 + 10 mg de caolin met en évidence les mêmes faits.

L'injection du caolin pur en doses progressives de 10, 20, 40 mg n'a pas produit dans la rate et le foie des animaux aucune lésion évolutive du type des nodules silicotique. L'observation des cas jusqu'à 18 mois après l'injection n'a montré que des petits et rares groupements isolés de celules dans quelques

espaces de Kiernan, sans tendance à la fibrose progressive. La surface occupée par ces cellules en rapport avec celle occupée par le tissu sain est minime. L'évolution dans le temps est presque inexistente entre 6 mois et 18 mois, et les différences quantitatives entre les doses expérimentales sont petites (occupant seulement quelques pour cent de la surface étudiée).

Nos expériences obtenus par les deux méthodes ont ainsi donné des résultats semblables en ce qui concerne l'action du caolin pur. Ces résultats semblaient être discordants avec les résultats de certain auteurs (Rüttner) qui ont expérimenté l'action du caolin pur en l'introduisant par voie intrapéritonéale. Les auteurs ont conclu que la poussière de caolin avait une action fibrosante similaire à celle de la SiO_2.

Nous avons également injecté, dans le péritoine des animaux expérimentaux, du caolin pur (50 mg) aussi que, chez un lot témoin, 50 mg de SiO_2 cristalline dans la dispersion mentionnée plus haut. Deux mois après l'introduction intrapéritoneale du caolin, l'on obtient des nodules sur l'épiploon, sur le mésentère et les anses intestinales, semblables à ceux provoqués par la SiO_2 pure. A l'examen histologique on observé une capsule assez importante de tissu conjonctif, un phénomène semblable à celui provoqué par le bioxyde de silicium. L'évolution est toutefois plus lente. Les ganglions mésentériques n'arrivent pas à développer des lésions fibrotiques caractéristiques pour les poussières contenant de la SiO_2 ni même après 12 mois et plus, ils sont peu hypertrophiés et seulement après 12 mois l'on observe quelques groupements de cellulles à poussières, preuves seulement de la migration cellulaire, sans d'autre signe d'évolution.

Cette expérience prouve la rélativité des résultats obtenus par la méthode des injections péritonéales, même dans le cas de l'expérimentation des silicates purs.

En ce qui concerne l'action du caolin sur l'évolution de la silicose, nos expériences ont montré son action cumulative par les deux méthodes.

Les dates de la littérature (Gardner, Schepers, King) montrent que l'empoussièrage par des mélanges, après plusiers années d'expériences, produit seulement des pneumoconioses bénignes avec une très lente évolution, compatible avec la vie quasi-normale de l'animal d'expérience. Après 3—4 années d'empoussièrage avec des poussières de granite qui contient 30% de la SiO_2, Gardner n'a pu obtenir que des rares nodules silicotiques, et au cours de 2 années la réaction était surtout alvéolaire.

Il était également nécessaire d'établir par cette méthode l'effet cumulatif du caolin.

Dans notre expérience on a empoussièré simultanément et avec la même quantité de quartz deux lots d'animaux. Le deuxième lot recevait simultanément une dose à peu près trois fois plus grande de caolin. On a effectué ainsi dans une des cabines un empoussièrage de 7 à 10 mille particules de SiO_2 par cm^3 d'air et dans l'autre cabine 7 à 10 mille particules de SiO_2 + 40 à 50 mille particules de caolin. Le système d'empoussièrage utilisait des distributrices à vis d'après le modèle de l'auteur soviétique Chakbazian, avec quelques modifi-

cations. Les variations de l'empoussièrage à de la SiO_2 s'effectuaient simultanément dans tous les deux cabines, parce que le distributeur de la poussière de SiO_2 était unique; c'est pourquoi l'on peut supposer que les deux lots d'animaux ont inspiré la même quantité de SiO_2 (fig. 2).

Dans l'expérience on a utilisé également des rats blancs qui ont été empoussiérés chaque jour pendant 6 heures et sacrifiés après 7 jours, 30 jours, 60 jours, 4 mois, 6 mois, 9 mois, 15 mois et 22 mois. Chez tous les rats sacrifiés on a fait l'examen histologique des poumons et des ganglions médiastinaux d'après les mêmes critères que dans les expériences précédantes.

Chez les animaux sacrifiés pendant les premiers mois de l'expérience, l'alveolite présente la même image comme chez l'instillation — un changement essentiel des poumons. Quoique, dans la cabine contenant un mélange de poussières, la quantité de poussières ait été 4 fois plus grande et que l'alvéolite ne représent pas une réaction spécifique aux poussières, l'étendue de l'alvéolite n'était pas plus grande sous l'influence du bioxyd de silicium mélangé au caolin, que sous l'influence du bioxyde de silicium pur.

Chez les animaux sacrifiés après quatre mois, on observe le début de la formation des futures nodules silicotiques. En certaines zones, on voit que les septums interalvéolaires se sont épaissis et qu'il y a une accumulation de cellules à poussière qui remplissent les alvéoles de cette zone. Beaucoup de phagocytes ont un aspect globulaire avec le protoplasme clair contenant parfois un petit noyau. Les septums sont modestement infiltrés par des cellules à poussière avec un aspect vieilli et contenant un noyau tachychromatique, mais peu de protoplasme. Ni dans cette phase il n'y a pas des différences significatives entre les deux lots parallèls.

Après 9 mois, le stade de petits nodules cellulaires périvasculaires ou même indépendants des vaisseaux et des bronches est caractéristique. Il y a un réticulum relativement rare de réticuline, qui présente, quelquefois, une métaplasie collagène près des parois alvéolaires compris dans les conglomérations cellullaires. Les ganglions hypertrophiés contiennent une conglomération importante de cellules à poussière dans un réticulum de réticuline. Il n'y a pas de différences entre les lots ayant reçu du caolin mélangé avec le quartz et celles ayant reçu du quartz pur, ni en ce qui concerne la fibrose périvasculaire modérée, ni en ce qui concerne la hypertrophie du ganglion ou le réticulum de réticuline. La différence entre les deux lots réside surtout dans l'étendue des lésions qui sont un peu plus nombreuses que celles provoquées par la SiO_2 pure mais la différence n'est pas importante.

En commençant avec cette étape, nous avons enlevé de leurs cabines une partie des rats des deux lots et nous les avons laissé respirer dans l'air pur. En conséquence, dans l'etape suivante — après 15 mois du début — nous avons pu comparer 4 lots d'animaux, dont deux (avec et sans caolin) avaient été soumis seulement à 9 mois d'empoussièrage, mais totalisaient 15 mois de la maladie.

Après 15 mois, les différences entre les deux lots avec du caolin et de la SiO_2 en comparaison avec celui avec de la SiO_2 pure sont impréssionnantes. Les

lésions sont 3—4 fois plus grandes, et cette tendance s'accentue chez les animaux qui ont subi 15 mois d'empoussièrage continu. En dehors de grands nodules placés en couronne et occupant une zone large de la périférie du poumon observés chez les deux lots qui ont reçu du caolin, ceux qui avaient été empoussiérés continuellement pendant 15 mois les nodules sont réunis par des zones d'alvéolite macrophagique importante.

Dans la zone périférique, les nodules ont un réticulum bien net de fibres collagènes, quelques-uns possédant en outre, dans l'étoile centrale, la métaplasie collagène. Les modifications bronchitiques sont beaucoup plus importantes que celles observées chez les animaux ayant reçus la SiO_2 pure. La fibrose provoque un rétrécissement du diamètre intérieur de la bronche, comme aussi un abaissement de l'élasticité; l'épithèle cylindrique de la bronche se hypertrophie, se décolle sur de grandes étandues, en rétrécissant beaucoup le diamètre intérieur des grandes bronches. Aussi les ganglions sont-ils plus grands et plus fibrosés que ceux provoqués par la SiO_2 pure.

Les zones occupées par les nodules et l'alvéolite en évolution occupent plus de 50% de la surface des sections pulmonaires, en comparaison avec 10—15% occupée par les nodules chez le lot avec la SiO_2 pure. Les nodules chez les rats témoins sont beaucoup plus rares et plus petits, mais ils contiennent aussi un réticulum riche de fibres collagènes.

L'empoussièrage a été poursuivi au cours de 18 mois et la sacrification des derniers animaux a été faite après 22 mois. A cette époque il n'y avait plus aucun animal vivant dans les lots soumis à l'influence du mélange de SiO_2 avec caolin, tous ayant péri à cause des lésions très étendues. Chez les animaux soumis à l'influence de la SiO_2 pure, une silicose pseudo-tumorale s'est developpée avec des nodules presque complètement hyalinisés, occupant des zones entières de chaque lobe avec une fibrose très avancée. On observe des modifications bronchiques atrophiques et des fibroses périvasculaires importantes qui exercent une influence sur les parois des vaisseaux.

CONCLUSIONS

Dans toutes les méthodes expérimentales employées, la caolin a produit un agrandissement des lésions silicotiques, proportionnel à la quantité administrée et au temps écoulé à partir du début de l'expérience. Le caolin seul, administré indifféremment par instillations ou par injection intraveineuse, a produit des changements minimes, sans tendances évolutives (leur augmentation ou leur métaplasie fibreuse).

Les résultats obtenus par l'injection intrapéritonéale, ne sont pas concordants étant également réfutés par l'évolution bénigne des modifications ganglionnaires chez tous les lots, même chez ceux où l'on a pratiqué l'introduction intrapéritonéale. Toutes les autres méthodes ont apporté des résultats concordants.

Les données cliniques confirment l'action cumulative du caolin, surtout quand il est inhalé en grande quantité.

Linch et Mc Iver donnent les détails des modifications pulmonaires chez des travailleurs qui se sont trouvés en contact de plusieurs années avec les poussières de caolin. Les auteurs ont examiné les poumons de 2 travailleurs, employés dans les entreprises s'occupant de l'extraction et préparation du caolin et décédés à la suite de la pneumoconiose. Chez un de ces hommes, qui y avait travaillé 17 ans, on a trouvé, dans le poumon et les ganglions, une augmentation importante du tissu fibreux interalvéolaire péribronchique ainsi que des épaissements pleurals.

L'autre cas était âgé de 35 ans et avait travaillé 21 années à la preparation du caolin. On a trouvé, chez lui, des portions massives de la fibrose pulmonaire, la déformation des vaisseaux et l'épaisissement de la plèvre. Les ganglions lymphatiques contenaient de grandes portions de fibrose collagène. L. W. Hale, J. Gough, F. J. King, M. Nagelschmidt on communiqué les données cliniques et radiologiques de 6 cas avancés de caolinese de Cornwale. Un cas arrivé à la nécropsie a été comparé avec un autre cas de Georgia (U.S.A.). Les découvertes post mortem ont démontré de très grandes quantités de caolin (20—40 g) dans les deux poumons, avec une petite concentration de la SiO_2 cristalline.

RÉSUMÉ

L'expérience a été entreprise pour tester l'action de quelques silicates argileux, caolin et montmorillonite, sur l'évolution de la silicose.

On décrit les changements histologiques des organs suivants: poumons, foie, rate, peritoine, nodules lymphatiques, observés après 11 jusqu'à 15 mois de l'instillation intratrachéale, de l'injection intraveineuse et intrapéritonéale et de l'empoussièrage de divers lot de rats blancs.

Le caolin pur ne produit pas des lésions après l'instillation intratrachéale et l'injection intraveineuse. Dans le péritoine se produissent, pourtant, de nodules fibreux sans que les nodules lymphatiques soient affectés.

Les mélanges des doses variables de SiO_2 et de caolin ont provoqué, par toutes les méthodes utilisées, une action cumulative du caolin.

SUMMARY

An experiment was carried out on several groups of white rats to study the effect of certain aluminium silicates, kaolin and montmorillonit, on the development of silicosis.

Histological changes in the following organs are described: lungs, liver, spleen, peritoneum and lymphatic nodes, which were observed for 11 and even 15 months after treatment. The preparations were administered either by pouring into the trachea, by intravenous injection or by inhalation of dust.

Pure kaolin does not produce lesions in tissue after being poured into the trachea or injected into the vein. However, fibrous nodules form in the peritoneum without the lymphatic nodes being affected.

Nevertheless, using mixtures of different doses of silicon dioxide and kaolin revealed an enhancing effect of kaolin with each of the methods of application employed.

ZUSAMMENFASSUNG

Man hat einen Versuch unternommen, die Wirkung einiger Aluminiumsilikate, Kaolin und Montmorillonit, auf die Entwicklung der Silikose zu untersuchen.

Gleichzeitig beschreibt man die histologischen Veränderungen an Lungen, Leber, Milz, Bauchfell und lymphatischen Knötchen, die nach 11 bis 15 Monaten der intratrachealen Einträufelung, intravenösen und intraperitonealen Einspritzung und Verstaubung an verschiedenen Gruppen von weißen Ratten beobachtet wurden.

Der reine Kaolin ruft keine Beschädigungen nach intratrachealer Einträufelung und intravenöser Einspritzung hervor. Im Bauchfell entwickeln sich jedoch faserige Knötchen, ohne daß die lymphatischen Knötchen in Mitleidenschaft gezogen werden.

Verschiedene Mischdosen von Siliziumdioxyd und Kaolin haben bei allen angewandten Methoden die kumulative Wirkung von Kaolin offenbart.

RESUMEN

Se emprendió un intento para examinar el efecto de algunos oxidos de aluminio, caolin y montmorilonit, sobre el desarrollo de la silicosis.

Se describen cambios histologicos en pulmón, hígado, bazo, peritoneo, nudolos linfaticos, observados después de 11 hasta 15 meses de instilación intratraqueal, inyección intravenosa y intraperitoneal y espolvorización de diversos grupos de ratas blancas.

El caolin puro no produce lesiones después de la instilación intratraqueal y la inyección intravenosa. En el peritoneo se producen, sin embargo, nodulos fibrosos sin que los nodulos linfaticos sean lesionados.

Mezclas de diversas dosis de SiO_2 y de caolin han provocado un efecto acumulativo de caolin en todos los metodos utilizados.

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Fatty Degeneration of the Liver Under the Influence of Bentonite and Lipotropic Effect of Intestinal Mucosa

E.N. Vasil'eva

Laboratory of the Physiology and Pathology of Digestion (Head -- Prof. G. K. Shlygin) of the Institute of Nutrition of the AMN SSSR (Academy of Medical Sciences of the U.S.S.R.), Moscow

It is known that a lipotropic effect on the liver is manifested by some substances found in food (choline, methionine, inositol, and others), and also, by internal lipotropic factors that are manufactured by the digestive system. Among the latter belongs the lipotropic factor of the pancreas and, as has been indicated by investigations in our laboratory, the substance produced by the mucous membrane of the small intestine (1, 2).

In connection with the existence of a close bond between the intestine and the liver, particular interest may be found in the influence manifested on the liver by the experimental adsorption of some substances, in particular, lipotropic ones, in the intestine. For such an experimental adsorption use may be made of bentonite -- a special clay, one of whose properties is the capacity to be metabolized both by inorganic, as well as by organic, bases. In particular, in experiments in vitro, it has been shown that bentonite can combine various amines (3, 4); among them, as is known, belongs choline, too.

In connection with this property, bentonite was utilized previously by Wilson who had observed in mice the development of a fatty infiltration of the liver in 2 months following the addition of bentonite to the control diet(5,6).

Proceeding from the properties of bentonite, it is possible to assume that it adsorbs, in the intestine, various organic substances, among which may be both food choline, as well as internal dipotropic factors. At the same time, if, in the action of bentonite, there predominates the adsorption of exogenous lipotropic substances, the nutritional background should manifest an influence on the periods of development of fatty infiltration. For this reason, it was

interesting to clarify during which periods there develops a fatty degeneration of the liver under the influence of bentonite, in the case of diets with varying amounts of albumin (protein), and consequently, lipotropic food substances.

The absence of such distinctions in the periods of development of the fattening will testify to the significance, in this effect, of internal lipotropic factors. In this case, it may be expected that the administration of a preparation of an intestinal secretion will manifest a positive therapeutic effect.

Procedure

In our experiments we made use of natural Uzbek bentonite. The tests were carried out on male mice, weighing 18 - 21 grams, of the C57BL, C3N and C3NA lines. The control animals in the 1st series of experiments received, over a period of 7 days, 1 of 2 diets with the following composition: 1) the control diet contains, relative to caloricity, 20% casein, 50% saccherose, 18% lard, and 2% fish fat. To this mixture was added, by weight, 4% of a Jones and Foster salt mixture, and 3% dry brewer's yeast. 2) the protein-deficient diet contained 6% protein, 74% saccharose, and for the remainder, the same as in the control diet.

The experimental animals received the abovementioned diet with an addition of bentonite comprising 30% in the overall weight of the diet. The food and water were given ad libitum. The weight of the mice was checked twice a week.

At the end of the experiment, the animals were put to death by means of decapitation, and a determination was made of their fat content in the liver tissue connected together from 5 animals. All the data are set forth figuring on dry tissue of the liver.

Results of the Investigations

In all the experiments, the mice, which had received bentonite, added less weight than the control animals; some of them even lost, in 7 days, up to 25%

of the initial weight. Nevertheless, independently of the change in weight, in all cases, the introduction of bentonite with food, in the course of a week, brought about an increase in the fat content in the liver, in various experiments, from 24 to 158%. Such comparatively large fluctuations were associated with the varying initial fat content in the liver of mice (we were able to judge this from the fat content in the liver of the control animals). The amount of fat in the liver in various groups of the control mice fluctuated within the limits of 11.1 - 20.6%; on the average, this comprised 15.2 ± 4.5%. In mice that had received bentonite, the fat content in the liver increased up to 14.8 - 40.1% (M = 25.4; 6 ± 9.4%). At the same time, a more significant increase in the fat (by 110 - 158%) was noted in experiments with a low initial fat level in the liver (11.1 - 15.5%). With a higher fat content in the liver of the control animals (20%), bentonite produced a lesser effect (an increase by 24 - 30%).

((to left of graph on pg. 72 of text)): Fat content; ((at bottom)): a; b;, v; g; ((below)): Fig. 1 Fat content in the liver of mice belonging to the C3NA line (in % relative to the weight of dry tissue) that had received, over a period of 7 days, a control diet (a), a control diet with 30% bentonite (b), a protein-deficient diet (v), and a protein-deficient diet with 30% bentonite (g).

((text continued)) In order to clarify in what minimal periods there is a change in the fat content in the liver, there were additionally set up 3-, 4-, and 5-day experiments. These investigations indicated that after 3, and sometimes, even after 5 days, the fat content in the liver of mice that had received bentonate was even lower than that in the control animals; this, apparently, is associated with the fact that in the first days the experimental animals ate less food than did the control ones. It is only after 7 days that the fatty degeneration of the liver was observed in all instances.

Similar results were obtained also in experiments with protein-deficient

diets. A protein-deficient diet, as is known, may, by itself, bring about a fatty degeneration of the liver. Following an experiment that had lasted 4 - 7 days, the fat content in the liver of mice that had received a protein-deficient diet increased negligibly, comprising, on the average, 18.6 ± 6.6%. The addition of bentonite to a protein-deficient diet, in all experiments, brought about an additional increase in the amount of fat in the liver of up to 36.5 ± 9.2%. The average increase in the fat content in the liver, in these experiments, comprised 126%. At the same time, between the degree of fat increase and its initial level in the liver, there was manifested the same inverse dependence as in the addition of bentonite to a diet with a normal protein content. However, on the average, against a background of a protein-deficient diet, an increase in the amount of fat, under the influence of bentonite, was more significant (126% in 7 days against a background of a protein-deficient diet, and 72% against a background of control diets). Apparently, upon introducing bentonite with a protein-deficient diet, there is manifested a total action on the part of bentonite and in that of a protein shortage (Fig. 1).

((to left of graph on pg. 73 of text)): Fat content; ((at bottom)): b;v;a.

((below)): Fig. 2 Fat content in the liver of mice belonging to the C57BL line with the action of 20 mg of an acetone preparation of the mucous membrane from the intestine of pigs (a) in control mice, without the introduction of the preparation (b), and with the introduction of 20 mg of starch (v).

((text continued)): In feeding mice with a protein-deficient diet with bentonite in the course of the first 5days, an increase in the amount of fat in the liver, as a rule, was not observed. On the contrary, in the first 3 days (and sometimes, even in 5 days), the fat content, as in the case of a control diet, decreased, but after 7 days, it was always increased.

In this manner, the investigations, which had been carried out with varying diets, indicated that the introduction, with food, of bentonite, brings about, in all the mice, an increase in the fat content in the liver after 7

days, independently of the protein content in the diet. We believed it possible to verify the action of the intestinal lipotropic factor on such animals.

As a lipotropic agent source use was made of scrapings from the mucous membrane of pigs' small intestines that have been dried by means of repeated treatment with acetone. Scrapings treated in this manner were stored prior to the experiment under acetone in a refrigerator. A suspension of 20 mg of a dry acetone preparation, in 0.25 ml of a physiological solution was administered to mice with a probe at the end of the 8th day of the experiment following a 7-hour period of hunger (to the end of the experiment the animals received only water).

The mice were put to death after 17 hours following the administration of the preparation and a study as made of their liver for the fat content. Similar mice served as a control; instead of the preparation, they were administered 20 mg of starch or only 0.25 ml of the physiological solution.

The lipotropic activity of the preparation was expressed in a reduction of the fat content in the liver in percents relative to its content in the liver of the control animals taken as 100.

In experiments with various diets, a verification was made of the action of 4 acetone preparations from the mucous membrane of pigs' intestines, and also, the action of 10 mg of methionine; the therapeutic activity of the latter, in fatty degeneration of the liver, is known.

The results of these experiments showed that intestinal secretion manifests a lipotropic effect in fatty degeneration of the liver brought about by bentonite. The preparations obtained from the pigs, in 4 experiments, brought about a reduction in the fat content in the liver by 18 - 30%, on the average, by 22%, in comparison with the control (Fig. 2). In 2 other experiments, similar preparations did not manifest any lipotropic effect. T he action of methionine, taken for comparison, was significant. It reduced the fat content by 27 and

30% (Fig. 4). It should be taken into consideration, however, that the dose of methionine was very high in comparison with the small amount effective in the beginning, which is found in 20 mg of an unpurified preparation of the mucous membrane in the intestine (methionine, in this amount of the preparation, is found to be close to 0.5 mg).

The development of fatty degeneration of the liver upon the administration of bentonite, and also, a diminution in the amount of fat during the administration of an acetone preparation, were likewise confirmed histologically.

In Fig. 3 it is shown that the administration of bentonite brings about a diffused, fine- and large-drop fatty infiltration of the liver. Upon administering an acetone preparation of the mucous membrane from a pig.s intestine, the fatty infiltration is maintained along the periphery of the lobules; the central part of the lobules is free of histologically visible fat (Fig. 4).

Our investigations have shown that the introduction, with food, of 30% bentonite, in the course of 7 days, brings about a fatty degeneration of the liver in mice. This attests to the fact that bentonite adsorbs, in the intestine, some highly important substances; a shortage of the latter the liver is not capable of compensating even in the course of a short period.

The nature of the compounds that are adsorbed in the intestine has not been established. The fact, however, that the fatty degeneration of the liver, under the influence of bentonite, develops during the same periods, with varying protein contents in the food, as well as that of lipotropic substances, attests to the considerable part played, in this process, by the internal lipotropic agents. It has not been excluded that among them the intestinal lipotropic factor finds significance. The positive effect, which has been brought about in a number of cases by the administration of intestinal secretion preparations confirms this assumption.

Further studies are necessary for the definitive solution of this problem.

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РАЗВИТИЕ ОЖИРЕНИЯ ПЕЧЕНИ ПОД ВЛИЯНИЕМ БЕНТОНИТА И ЛИПОТРОПНОЕ ДЕЙСТВИЕ СЛИЗИСТОЙ КИШЕЧНИКА

Э. Н. Васильева

Лаборатория физиологии и патологии пищеварения (зав. — проф. Г. К. Шлыгин) Института питания АМН СССР, Москва

Известно, что липотропное действие на печень оказывают некоторые вещества, содержащиеся в пище (холин, метионин, инозит и др.), а также внутренние липотропные факторы, вырабатываемые пищеварительной системой. К последним относится липотропный фактор поджелудочной железы и, как показали исследования нашей лаборатории, вещество, продуцируемое слизистой оболочкой тонкого кишечника [1, 2].

В связи с существованием тесной связи между кишечником и печенью особый интерес может представлять влияние, оказываемое на печень экспериментальной адсорбцией некоторых веществ, в частности липотропных, в кишечнике. Для такой экспериментальной адсорбции может быть использован бентонит — особая глина, одним из свойств которой является способность обмениваться как неорганическими, так и органическими основаниями. В частности, в опытах in vitro было показано, что бентонит может присоединять различные амины [3, 4], к которым, как известно, относится и холин.

В связи с этим свойством бентонит был использован ранее Вильсоном, наблюдавшим у мышей развитие жировой инфильтрации печени через 2 месяца после добавления бентонита к контрольному рациону [5, 6].

Исходя из свойств бентонита, можно предполагать, что он адсорбирует в кишечнике различные органические вещества, в числе которых может быть и холин пищи, и внутренние липотропные факторы. При этом если в действии бентонита преобладает адсорбция экзогенных липотропных веществ, фон питания должен оказывать влияние на сроки развития жировой инфильтрации. Поэтому представляло интерес выяснить, в какие сроки развивается ожирение печени под влиянием бенто-

нита при диетах с разным содержанием белка, а следовательно, и липотропных веществ пищи.

Отсутствие таких различий в сроках развития ожирения будет свидетельствовать о значении в этом эффекте внутренних липотропных факторов. В этом случае можно ожидать, что введение препарата кишечного секрета окажет положительное терапевтическое действие.

Методика

В наших опытах мы использовали природный узбекский бентонит. Исследования проводили на мышах-самцах весом 18—21 *г*, линий C57BL, C3H и C3HA. Контрольные животные в I серии опытов получали в течение 7 дней 1 из 2 рационов следующего состава: 1) Контрольный рацион содержал по калорийности 20% казеина, 60% сахарозы, 18% лярда и 2% рыбьего жира. К этой смеси добавляли по весу 4% солевой смеси Джонса и Фостера и 3% сухих пивных дрожжей. 2) Малобелковый рацион содержал 6% белка, 74% сахарозы и в остальном то же, что и контрольный рацион.

Подопытные животные получали описанные выше рационы с добавлением бентонита, составлявшим 30% в общем весе рациона Пищу и воду давали ad libitum. Вес мышей проверяли дважды в неделю.

В конце опыта животных забивали путем декапитации и определяли у них содержание жира в ткани печени, соединенной от 5 животных. Все данные приводятся из расчета на сухую ткань печени.

Результаты исследований

Во всех опытах мыши, получавшие бентонит, прибавляли в весе меньше, чем контрольные животные; некоторые из них даже потеряли за 7 дней до 25% первоначального веса. Тем не менее независимо от изменения веса во всех случаях введение с пищей бентонита в течение недели вызвало увеличение содержания жира в печени в разных опытах от 24 до 158%. Такие сравнительно большие колебания были связаны с разным исходным содержанием жира в печени мышей (об этом мы судили по содержанию жира в печени контрольных животных). Количество жира в печени разных групп контрольных мышей колебалось в пределах 11,1—20,6%, что в среднем составляло 15,2±4,5%. У мышей, получавших бентонит, содержание жира в печени повысилось до 14,8—40,1% (M=25,4; 6±9,4%). При этом более значительное увеличение жира (на 110—158%) отмечалось в опытах с низким исходным уровнем жира в печени (11,1—15,5%). При более высоком содержании жира в печени контрольных животных (20%) бентонит вызывал меньший эффект (увеличение на 24—30%).

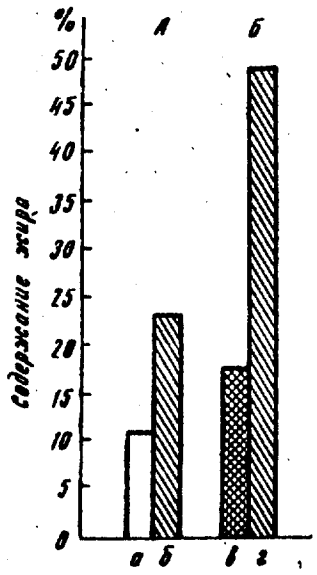

Рис. 1. Содержание жира в печени мышей линии C3HA (в % к весу сухой ткани), получавших в течение 7 дней контрольный рацион (*а*), контрольный рацион с 30% бентонита (*б*), малобелковый рацион (*в*), малобелковый рацион с 30% бентонита (*г*).

Для того чтобы выяснить, в какие минимальные сроки изменяется содержание жира в печени, были дополнительно поставлены 3-, 4- и 5-дневные опыты. Эти исследования показали, что через 3, а иногда и через 5 дней содержание жира в печени мышей, получавших бентонит, было даже более низким, чем у контрольных животных, что, по-видимому связано с тем, что в первые дни экспериментальные животные съедали меньше пищи, чем контрольные. Лишь через 7 дней ожирение печени отмечалось во всех случаях.

Подобные результаты были получены и в опытах с малобелковым рационом. Малобелковый рацион, как известно, может сам вызывать ожирение печени. После опыта, длившегося 4—7 дней, содержание жира в печени мышей, получавших малобелковую диету, повысилось незначительно, составляя в среднем 18,6±6,6%. Добавление к малобелковому рациону бентонита во всех опытах вызвало дополнительное повышение количества жира в печени до 36,5±9,2%. Среднее повышение содержания жира в печени в этих опытах составляло 126%. При этом между степенью увеличения жира и его исходным уровнем в печени проявлялась та же обратная зависимость, что и при добавлении бентонита к рациону с нормальным содержанием белка. Однако в среднем на фоне малобелкового рациона увеличение количества жира под влиянием бентонита было более значительным (126% за 7 дней на фоне малобелкового рациона и 72% на фоне контрольных рационов). По-видимому, при введении бентонита с малобелковой диетой проявляется суммарное действие бентонита и недостатка белка (рис. 1).

При кормлении мышей малобелковым рационом с бентонитом в течение первых 5 дней увеличения количества жира в печени, как правило, не наблюдалось. Напротив, в первые 3 дня (а иногда и 5 дней) содержание жира, как и на контрольном рационе, уменьшалось, но через 7 дней оно всегда было повышенным.

Таким образом, исследования, проведенные с разными рационами, показали, что введение с пищей бентонита вызывает у всех мышей увеличение содержания жира в печени через 7 дней независимо от содержания белка в рационе. На таких животных мы считали возможным проверить действие кишечного липотропного фактора.

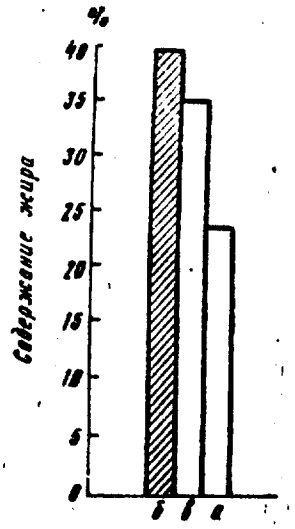

Рис. 2. Содержание жира в печени мышей линии C57 BL при действии 20 *мг* ацетонового препарата слизистой оболочки кишечника свиней (*а*) у контрольных мышей без введения препарата (*б*) и при введении 20 *мг* крахмала (*в*).

В качестве источника липотропного агента использовались соскобы слизистой оболочки тонкого кишечника свиней, высушенные путем многократной обработки ацетоном. Обработанные таким образом соскобы хранились до опыта под ацетоном в холодильнике. Взвесь из 20 *мг* сухого ацетонового препарата в 0,25 *мл* физиологического раствора вводили мышам зондом в конце 8-го дня опыта после 7-часового голода (до конца опыта животные получали только воду).

Мышей забивали через 17 часов после введения препарата, и исследовали их печень на содержание жира. Контролем служили такие же мыши, которым вместо препарата вводили 20 *мг* крахмала или только 0,25 *мл* физиологического раствора.

Липотропная активность препарата выражалась в снижении содержания жира в печени в процентах к содержанию его в печени контрольных животных, принимаемому за 100.

В опытах с разными рационами было проверено действие 4 ацетоновых препаратов из слизистой оболочки кишечника свиней, а также действие 10 *мг* метионина, терапевтическая активность которого при ожирении печени известна.

Результаты этих опытов показали, что кишечный секрет оказывает липотропное действие при ожирении печени, вызванном бентонитом. Препараты, полученные у свиней, в 4 опытах вызвали снижение содержания жира в печени на 18—30%, в среднем на 22%, по сравнению с контролем (рис. 2). В 2 других опытах подобные препараты не оказали

липотропного действия. Действие метионина, взятого для сравнения, было значительным. Он снизил содержание жира на 27 и 30% (рис. 4). Следует, однако, учитывать, что доза метионина была весьма высокой по сравнению с тем малым количеством действующего начала, которое содержится в 20 *мг* неочищенного препарата слизистой оболочки кишечника (метионина в этом количестве препарата содержится около 0,5 *мг*).

Развитие ожирения печени при введении бентонита, а также уменьшение количества жира при введении ацетонового препарата были также подтверждены гистологически.

На рис. 3 показано, что введение бентонита вызывает диффузную мелко- и крупнокапельную жировую инфильтрацию печени. При введении ацетонового препарата слизистой оболочки кишечника свиней жировая инфильтрация сохраняется по периферии долек; центральная часть долек свободна от гистологически видимого жира (рис. 4).

Наши исследования показали, что введение с пищей 30% бентонита в течение 7 дней вызывает у мышей ожирение печени. Это свидетельствует о том, что бентонит адсорбирует в кишечнике какие-то весьма важные вещества, недостаток которых печень не в состоянии компенсировать даже в течение короткого времени.

Природа адсорбируемых в кишечнике соединений еще не установлена. Однако тот факт, что ожирение печени под влиянием бентонита развивается в одинаковые сроки при разном содержании в пище белка и липотропных веществ, свидетельствует о значительной роли в этом процессе внутренних липотропных агентов. Не исключено, что среди них имеет значение кишечный липотропный фактор. Положительное действие, вызываемое в ряде случаев введением препаратов кишечного секрета, подтверждает это предположение.

Для окончательного решения вопроса необходимы дальнейшие исследования.

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THE INFLUENCE OF CERTAIN DRIED FRUITS AND ADSORBING AGENTS ON THE INTESTINAL FLORA OF WHITE RATS

LOUIS WEINSTEIN
AND
JAMES E. WEISS
NEW HAVEN, CONN.

The suggestion made by Haas that celiac disease of infants may be overcome by including banana in the diet has led to considerable research on the subject. Various investigators in the field of pediatrics have confirmed his results and have enlarged the category of diseases in which the banana is a helpful adjunct to the diet. A review of the literature on this subject is not presented here: for such the reader is referred to a recent publication by Bogert.[1]

Many suggestions have been advanced regarding the mode of action of bananas. The astringent action, the high vitamin content, and the large amount of easily available carbohydrate in them have all been thought to be responsible for the results obtained in their use. One of the theories advanced to account for their beneficial action is that they help to establish a favorable change in the intestinal flora. It is with this phase of the problem that we wish to deal here. Arnold, Korando and Ryan[2] studied the effect of banana powder on the intestinal flora of white rats and concluded that no increase in the aciduric bacteria took place, even after prolonged feeding. We shall attempt to show that banana in the form of the dry powder changes the flora of white rats from a predominantly proteolytic type to one rich in L. acidophilus. Several other fruits were studied by us in an attempt to determine whether or not fruits in general have the property of inducing a high aciduric flora in the intestine.

FEEDING EXPERIMENTS WITH FRUIT

The apple and banana used in these experiments were administered in the form of a dry powder, which was supplied to us by a commercial firm that is engaged in its production. The raisins and prunes were the semi-dried variety purchased on the open market.

Mature white rats were employed in the experiments. They were placed on a diet of raw chopped beef and the feces examined every three days until no lactobacilli were demonstrable for a period of at least one week. The test substances were then added to the chopped beef and their effect on the

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From the Department of Bacteriology, Yale University.

1. Dietary Uses of the Banana in Health and Disease, New York.
2. J. Infect. Dis. 51: 556, 1932.

intestinal flora determined. Several rats were always kept as controls; they were fed only raw meat throughout the experiment.

The method of fecal examination was as follows: A sample of feces about the size of a pea was suspended in 5 cc. of water. Approximately 0.1 cc. of this suspension was plated and the plates incubated in an atmosphere containing 10% carbon dioxide at 37 C. for 48 hours. The plates were then examined for L. acidophilus. The medium used had the following composition:

Bacto-Difco Peptone—5 grams
Difco Peptonized Milk—10 grams
Difco Yeast Extract—5 grams.
Filtered Tomato Juice—200 cc.
Water—600 cc.
Difco Agar—15 grams

The p_H was adjusted to 7.5 before the final autoclaving at 120 C. for ten minutes. The final p_H was 6.6 to 6.7.

BANANA POWDER

Eight rats, all of which had been freed from L. acidophilus in the feces by being fed the raw beef diet, were used in this experiment. Three of the animals were fed 12 grams of meat plus 4 grams of banana powder, three were given 12 grams of meat plus 6 grams of the dried banana powder; the remaining two were fed 12 grams of meat with no added fruit powder.

Ten days after the first banana feeding the two groups of rats receiving the banana powder were found to harbor large numbers of viable L. acidophilus in the feces; the three animals receiving 4 grams of powder gave L. acidophilus plate count of 50%, 70% and 50%, respectively, while the three that had 6 grams of banana powder daily gave counts of 95%, 90% and 80%, of the total viable intestinal flora, respectively. The experiments were extended over a period of two or more weeks, during which time the lactobacilli in the feces continued at essentially the same high levels. The control rats (fed meat only) were at all times apparently free from L. acidophilus. It was observed also that the rats receiving banana powder passed a much larger volume of fecal material than the control animals, and that their feces were lighter in color and softer in consistency than those of the controls.

APPLE POWDER

The procedure in the experiments with the apple powder was the same as that followed in the banana feeding trials. This fruit was also used in 4 and 6 gram quantities. Twelve days after the beginning of this experiment the feces of three rats receiving 4 grams of apple powder in their diet gave L. acidophilus counts of 0%, 10% and 80%, respectively, while those receiving 6 grams of powder showed 80%, 20% and 60%, respectively, of the total viable fecal flora to be L. acidophilus. One week later the feces of the animals receiving 4 grams of apple powder revealed 40%, 50% and 40% viable L. acidophilus while those which were given 6 grams showed that L. acidophilus

constituted 85%, 60% and 70% respectively, of the total viable intestinal flora. In general, the same results were obtained during the two ensuing weeks. As in the case of the banana, the feces of the apple powder fed rats were more bulky, softer in consistency and lighter in color than those of the control animals, which at no time during this experiment showed any L. acidophilus in their feces.

RAISINS

Eight rats whose aciduric intestinal flora had been reduced to zero by meat feeding were used in this experiment. One group of three was fed 12 grams of meat plus 4 grams of raisins daily; another group of three received 12 grams of meat plus 6 grams of raisins. Two (control) rats were fed only raw chopped beef. Within one week all of the animals receiving raisins were were found to harbor L. acidophilus in their intestine. The rats receiving 4 grams of raisins gave L. acidophilus counts of 40%, 20% and 40%, while those which were fed 6 grams of the fruit harbored viable L. acidophilus to the extent of 85%, 70% and 70%, respectively of the total intestinal flora. The control rats showed no L. acidophilus in their feces. Continuation of the raisin feeding for one more week yielded the following results: in the group receiving 4 grams of raisins, L. acidophilus constituted 50%, 40% and 50% of the total viable fecal flora, while the group getting 6 grams gave L. acidophilus counts of 90%, 80% and 80%, respectively. The controls again harbored no L. acidophilus in their feces. The dejecta of the animals receiving raisins were of the same general character as those of the rats fed banana and apple powder.

PRUNES

The procedure used in this experiment was the same as that employed in the experiments described above. Prunes fed in 4 and 6 gram amounts were found to exert no visible influence on the intestinal flora. Continued feeding of prunes for four weeks did not cause the appearance of aciduric organism in the feces. The controls were also free from L. acidophilus during the entire period. The experiments were concluded at the end of four weeks.

EXPERIMENTS WITH ADSORBING AGENTS

Braafladt[3] studied the effect of kaolin on the intestinal flora of rats, dogs and human subjects and came to the conclusion that this mineral, when ingested, brings about a change in the intestinal flora to a point where L. acidophilus predominates over the other bacterial forms. This alleged property of kaolin, together with its great adsorptive powers generally, and its ability to reduce the number of bacteria in a suspension by carrying them down with it as it is precipitated slowly, were thought by this investigator to furnish an explanation for the beneficial action of kaolin in such intestinal diseases as cholera.

A search of the literature since 1923 revealed no experiments which corroborated or disproved Braafladt's statement regarding the influence of

3. J. Infect. Dis. 33: 434, 1923.

kaolin on intestinal bacteria. It was our purpose in this experiment to repeat Braafladt's work using, however, a medium more favorable for the growth of L. acidophilus than that used by him. It was thought desirable also to employ another adsorbing agent, in order to determine if positive results, should they be obtained, are due to the adsorptive properties of the test substance or to its ability to act as pabulum for the aciduric organisms. Charcoal was the other adsorbing agent employed.

KAOLIN

Eight rats were used in the experiment. One group of three was fed 12 grams of meat plus 2 grams of kaolin daily; another group of three received 12 grams of meat plus 4 grams of kaolin, while two rats were fed 12 grams of meat alone. Fecal examination over a period of four weeks showed no viable L. acidophilus in any of the rats receiving kaolin; the same was true of the controls.

CHARCOAL

Eight rats were divided into three groups. The first three were fed 12 grams of the meat plus 2 grams of wood charcoal; the second group of three received 12 grams of meat plus 4 grams of charcoal, while the two remaining animals were allowed to subsist on a daily diet consisting of 12 grams of meat, with no added adsorbing substance. As in the case of the kaolin experiment, no viable L. acidophilus was demonstrable in the feces of any of the rats, even after four weeks.

DISCUSSION

The results of this investigation show that banana, apple and raisins are capable of establishing an intestinal flora that is high in L. acidophilus when they are fed in appreciable quantities to white rats. The reason for the failure of Arnold and his co-workers to report positive results with banana feeding must be obvious to anyone who had had any experience with L. acidophilus in its relation to other bacteria. Attempts to determine changes in intestinal flora by merely studying microscopic smears of the fecal material are futile, according to our experience. The most satisfactory procedure for obtaining a true picture of the viable bacterial content of feces is the cultural method. Since Arnold and his co-workers made no actual studies of the feces of their experimental animals, they probably did not get a true idea of the events taking place in the intestine. This, we believe, is the reason for the discrepancy between the results obtained by Arnold and his co-workers, and ourselves.

Why the feeding of banana, apple and raisin brings about the establishment of an intestinal flora high in L. acidophilus is not fully apparent at the present moment. Rettger and Horton[4] and other investigators have shown that diets high in lactose, dextrin or starch will cause an increase in acidurics, while glucose, maltose and sucrose do not have this effect. The ripe banana, according to analyses by various investigators, does not contain enough starch or dextrin to account for the results cited in the above experiments. However,

4. Centralbl. f. Bakt. (Abt. 1) O., 13: 362, 1914.

banana, apple and raisins do contain large amounts of mono- and di-saccharides, and it is possible that some of this carbohydrate material is not completely utilized and absorbed in the small intestine. Consequently it may descend into the colon and there serve as pabulum for L. acidophilus. These fruits also contain relatively large amounts of minerals and, in view of the work of Valley and Eppright[5] who have shown that the salt content of the diet plays an important role in the ability of carbohydrates to induce a highly aciduric flora, it may be possible that less of the starch or dextrin is required to bring about the desired result than when diets low in minerals are fed. The above-mentioned authors have shown that such a phenomenon is entirely possible. A study of the separate components of these fruits, singly and in combinations, will probably be necessary to furnish a satisfactory explanation of the mechanism involved. The reason for the failure to cause an increase in intestinal L. acidophilus with prune feeding is not at hand. One obvious difference between prune and the other fruits studied in this investigation is that the former is a so-called acid fruit, while the latter are basic fruits. The experiment with the prunes, we believe, tends further to show that the material composition of the fruit plays some part in its ability to bring about a highly aciduric intestinal flora, since the prune differs from the other fruits investigated in that it yields an acid ash, while the others produce an alkaline ash.

Braafladt believed that kaolin feeding induces an intestinal flora high in L. acidophilus because it adsorbs substances from the content of the colon which are inhibitory to the growth of this organism. Our experiments failed to corroborate his findings.

SUMMARY

Banana powder, apple powder and raisins, when fed to white rats subsisting on a high protein diet consisting exclusively of raw chopped beef cause a change in the intestinal flora from one in which non-aciduric bacteria predominate to one in which L. acidophilus is the outstanding organism.

Prunes, charcoal and kaolin do not have any visible effect on the intestinal flora of white rats.

The exact explanation for the action of banana, apple and raisins is not apparent, but it is quite probable that the carbohydrate and mineral contents of these fruits are of prime importance in their ability to establish an intestinal flora rich in L. acidophilus.

5. Yale J. Biol. & Med. 7: 563, 1935.

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J. Natl. Cancer Inst. 14:57-63. 1953.

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Nutritional Deficiency Produced in the Mouse by Feeding Bentonite [1,2]

J. WALTER WILSON,[3] *Arnold Biological Laboratory, Brown University, Providence, R. I.*

When mice were fed semisynthetic diets to which large amounts of bulk-forming material had been added, animals receiving a diet of 50 percent bentonite grew very slowly, developed fatty livers, and, after some months, eleven of twelve mice developed hepatomas [Wilson (*1*)]. Mice on similar diets with Ruffex or barium sulfate added showed good, if not normal, growth and their livers were normal. The similarity of the response of the bentonite-fed animals to that of mice in other experiments, fed on low-protein diets deficient in choline (*2*), suggested that these mice might also be subject to choline deficiency, possibly due to the fact that bentonite is a base-exchange silicate and thus removes choline from the contents of the intestine. The experiments herein discussed were undertaken to test this possibility.

Materials and Methods

The mice used were all of inbred strains BUA, BUB, or BUC, maintained in air-conditioned rooms at about 25° C. and fed routinely on Purina laboratory chow. The basal diet in these experiments is as follows: casein (Borden's "Edible") 20%, cornstarch (Argo) 40%, Crisco 20%, corn oil (Mazola) 4%, salt mixture (GBI-USP No. 2) 6%, yeast (GBI) 8%, and cod-liver oil (Squibb) 2%. This entire diet is mixed with the bulk-former in the required percentage by weight or, in some experiments, the bulk-former may replace a part of the cornstarch or Crisco. The bentonite was supplied by the American Colloid Company as "Volclay Bentonite." The montmorillonite was supplied by the Milwhite Company, Inc. The Ruffex is a pure cellulose material obtained from the Fisher Scientific Company, and the barium sulfate ("Baridol," a product especially prepared for gastroenterological roentgenography) was supplied by the Pacific Chemical Laboratories.[4]

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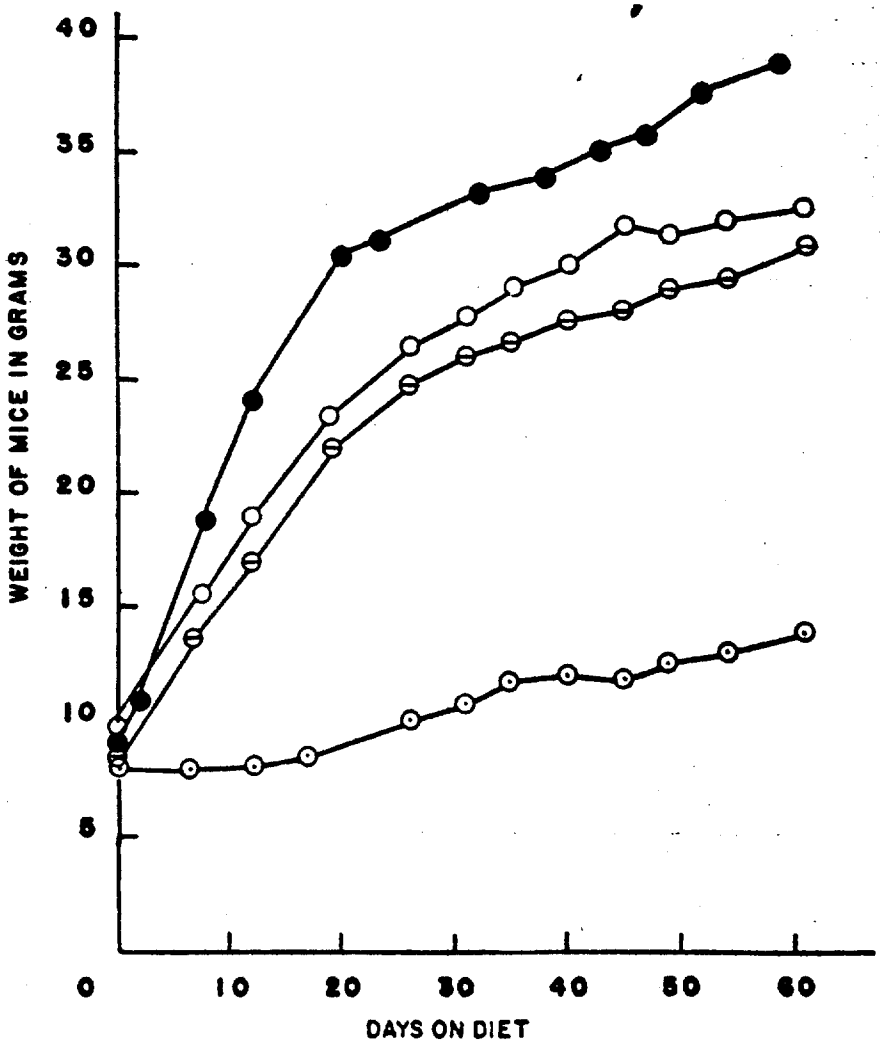
[2] Supported in part by grants from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, and from the Rhode Island Cancer Society.
[3] With the technical assistance of Miss Mary Day, Mrs. Harriet Parkhurst, and Miss Carolyn Swaney.
[4] Thanks are due to the following for generous supplies of materials: American Colloid Company, Chicago, Ill.; The Milwhite Company, Inc., Houston, Tex.; Pacific Chemical Laboratories, San Francisco, Calif.; and Merck & Co., Inc., Rahway, N. J.

57-63

All mice were weighed at least twice a week. At sacrifice, pieces of liver were fixed in Bouin's fluid, sectioned at 5 μ and stained with Delafield's hematoxylin and eosin. The liver was also fixed in 10-percent formalin and stained for fat with Sudan IV.

Experimental Results

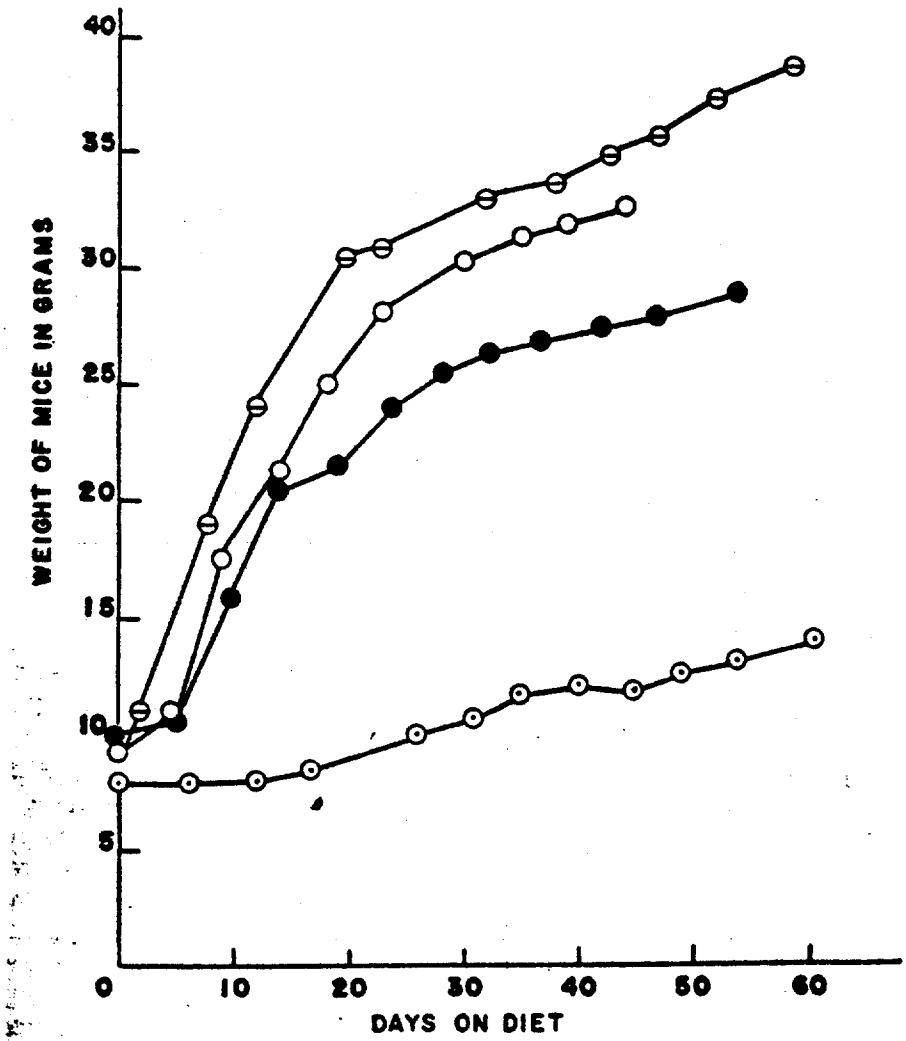
Two male and two female 18-day-old mice were placed on the basal diet and on diets with 10%, 25%, or 50% bentonite added to the basal diet (text-fig. 1) and maintained on these diets for 61 days. Those on the 10% and the 25% bentonite grew at a rate slightly below that of the controls,



TEXT-FIGURE 1.—Growth of male mice on the basal diet mixed with bentonite. Each curve represents the average weights of two mice placed on the diet at 18 days of age. ●—100% basal diet; ○—10/90 bentonite-basal diet; ⊖—25/75 bentonite-basal diet; ⊙—50/50 bentonite-basal diet.

but those on the 50% bentonite grew very slowly. The histologic sections of the livers of these retarded animals showed an abundance of fat vacuoles, whereas the livers of the other mice were normal.

When 50% Ruffex or 50% barium sulfate was used in place of the 50% bentonite in similar experiments (text-fig. 2), nearly normal increase in weight occurred, but when 50% montmorillonite (another clay related to bentonite) was used, the growth was extremely retarded. This indicates that the effect of the clay is not due to bulk alone and that it is not due to a contaminant in the bentonite unless the same contaminant occurs in a similar clay from a different source. The slightly lower rate of growth



TEXT-FIGURE 2.—Growth of male mice on diets containing 50% Ruffex, barium sulfate, or bentonite. Each curve represents the average weights of two mice placed on the diet at 18 days of age. ○—100% basal diet; ⊙—50/50 bentonite-basal diet; ●—50/50 Ruffex-basal diet; ⊖—50/50 barium sulfate-basal diet.

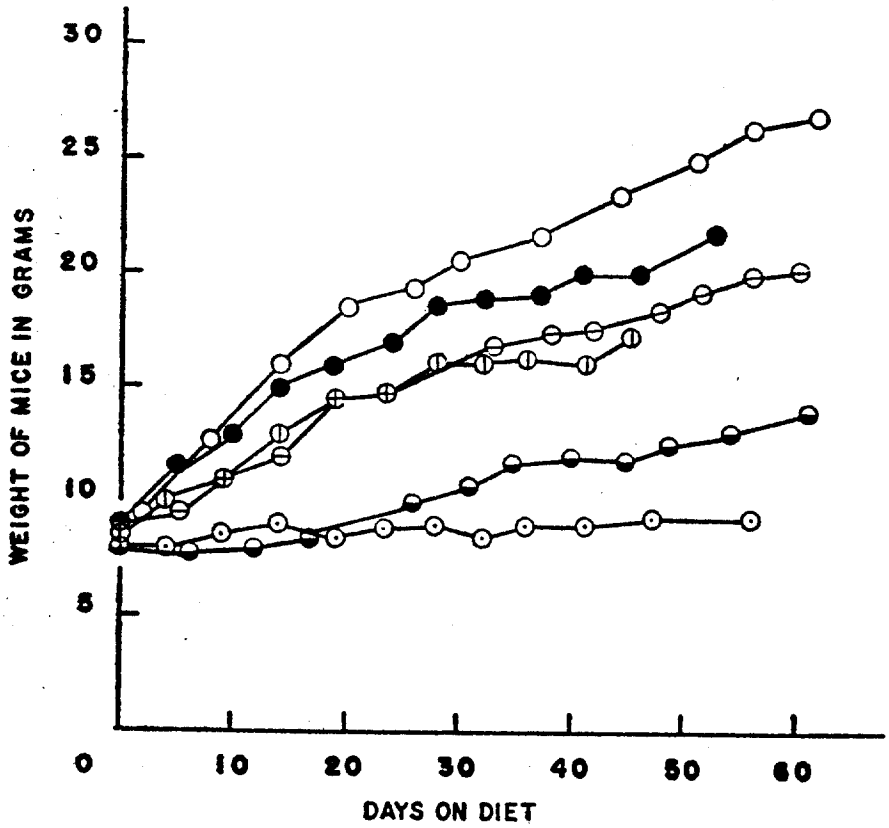
on Ruffex compared with barium sulfate may be attributed to the greater bulk of the former, which may result in inanition that may not be otherwise detectable.

The possibility that the extreme retardation on the 50-percent bentonite diet might be due to partial inanition was studied in an experiment in which for 16 days the food intake of two young adult (70-day-old) mice on each of the following diets was studied: basal, 50/50 bentonite-basal, and 50/50 Ruffex-basal. During this period the animals on the basal diet gained slightly in weight, those receiving 50 percent Ruffex held their weight, and those receiving 50 percent bentonite lost weight slightly. The average daily food intake per mouse for each group was as follows: basal, 3.37 gm.; 50% Ruffex, 5.92 gm.; and 50% bentonite, 6.14 gm. Since half the intake of the last two groups is inert material, the actual food intake was: basal, 3.37 gm.; 50% Ruffex, 2.96 gm.; and 50% bentonite, 3.07 gm. While the somewhat decreased food intake might account for the fact that the rate of growth on the 50-percent Ruffex diet is slightly less than on the basal diet, it does not explain the extreme retardation on the 50-percent bentonite diet.

A series of experiments was performed to counteract the retarding effect of the 50/50 bentonite-basal diet (text-fig. 3). When the bentonite was included in the diet, replacing 10%, 20%, or 30% of the cornstarch, all mice grew steadily at a nearly normal rate and their livers were normal. When 50% bentonite replaced 35% cornstarch and 15% Crisco, growth was moderately retarded, but not as completely so as on the 50/50 bentonite-basal diet, and the livers were normal. When the casein was doubled (vitamin-test casein was used so that unknown factors in the "edible casein" were not increased), or when the yeast, cod-liver oil, and salts were doubled at the expense of cornstarch, moderate growth occurred and the livers of the mice were normal. It would therefore appear that the retardation of growth and the fatty livers on the 50/50 bentonite-basal diet were not due to the bulk of the bentonite and consequent inanition, but to some nutritional deficiency.

The fatty livers, in addition to the retardation of growth on the 50/50 bentonite-basal diet, suggested a choline deficiency. When 0.15% choline chloride was added to the basal diet (text-fig. 3), moderate but not normal growth occurred and the livers were normal. On the other hand, when *d,l*-methionine, which should have a lipotropic action, was added at a level of 0.25% or 0.5% to the basal diet, the slight growth that occurred with the 50/50 bentonite-basal diet was suppressed, and at the 0.5% level none of the mice survived the 60-day test period. The methionine did not prevent fatty livers. Thus, while added choline and added casein correct to some extent the harmful effects of the 50/50 bentonite-basal diet, added methionine does not.

The possibility that a deficiency in choline is involved in the extreme retardation of growth suggested the use of a small amount of bentonite in the diet—low in protein and deficient in choline—used in our previous experiments on choline deficiency in the mouse [Wilson and Leduc (*3*)].



TEXT-FIGURE 3.—Growth of male mice on 50% bentonite with modified basal diets. Each curve represents the average weight of two mice placed on the diet at 18 days of age.

○—50% bentonite replacing 35% cornstarch and 15% Crisco in the basal diet.
●—50/50 bentonite-basal with doubled casein replacing half the cornstarch.
◐—50/50 bentonite-basal with doubled yeast, cod-liver oil, and salts replacing part of the cornstarch.
⊖—50/50 bentonite-basal with added 1.5 gm. choline chloride in 1,000 gm.
⊙—50/50 bentonite-basal with added 5 gm. *d,l*-methionine in 1,000 gm.
◒—50/50 bentonite-basal diet.

When placed on the low-protein, choline-deficient diet, weanling mice grew very slowly but frequently survived for many months and eventually developed hepatomas. When coramine, which is detoxified by transmethylation, was added to this diet the severity of the deficiency was increased, the weanling mice failed to grow, and survived for only short periods (*3*). When a diet of 10% bentonite and 90% of the low-protein, choline-deficient diet was used, the slight growth was also eliminated. The mice maintained their weight but did not thrive. The first one died on the 35th day and the last one was sacrificed on the 88th day because it seemed on the verge of death. The livers of all the mice were extremely fatty. It would seem, then, that even a small amount of bentonite, when added to a diet already deficient in choline, enhances the deficiency.

Discussion

Bentonite, far from being the inert material we had expected it to be, turned out to be a very active one. This activity is apparently due to its properties as a base-exchange silicate. Freundlich [(*4*) p. 34] states that bentonite may be considered to be a colloidal electrolyte with a very large micronic anionic micelle and small exchangeable cations. Because of the importance of this property in soil chemistry, it has been extensively studied by Grim (*5*) and Ross and Hendricks (*6*). Permutit, a synthetic base-exchange mineral, takes up choline and from it the choline is readily eluted [Whitehorn (*7*)]. Organic as well as inorganic cations may be involved in the base-exchange activity of bentonite and the larger ones are less easily removed, apparently because they are held by Van der Waals forces in addition to the Coulomb forces of the simple exchange [Hendricks (*8*)]. It is then possible that by its base-exchange capacity, bentonite may remove choline from the intestinal content stoichiometrically and hold it so that it is removed from the body in the feces. Whether or not this would occur in such a complex situation as the intestine could not, perhaps, be predicted because so many factors are involved, and furthermore it seems possible that other cationic materials might also be removed.

The experiments reported here would indicate that when substantial amounts of bentonite are included in the diet of the mouse, choline is removed and this determines the principal characteristics of the deficiency. There may be a slight tendency toward inanition when 50 percent of the diet is not food. When Ruffex or barium sulfate are used, growth is, at the most, only slightly inhibited and no other signs of deficiency appear. The occurrence of fatty livers in mice fed the 50/50 bentonite-basal diet, and not with the same diet when choline or protein are added, indicates choline deficiency. The addition of 10 percent of bentonite to the basal diet produces little or no effect, but when added to a diet low in protein and already deficient in choline, the slow growth that this diet permits is abolished. It would be difficult to explain these phenomena otherwise than by the removal of choline.

On the other hand, it is possible that some additional factor is involved in the deficiency. This seems clearly indicated by the failure of the added choline to restore growth completely to its normal level. It is also indicated by the complete failure of added methionine to produce growth or even to insure maintenance of body weight, for methionine should serve as a source for the labile methyl groups involved in choline deficiency. It is possible, for example, that pyridoxine, which is also a base, may be removed and that the large amount of added methionine enhances this deficiency to the point where it becomes a serious one. Another possibility is that removal of diamino acids by the bentonite produces an imbalance of amino acids which becomes a serious matter when methionine is added to the diet. Further experiments to test such possibilities are in progress.

Summary and Conclusions

Mice fed on a 50/50 bentonite-basal semisynthetic diet fail to grow and develop signs of choline deficiency, including fatty livers. This does not occur in animals fed a diet composed of 50 percent of either Ruffex or barium sulfate and 50 percent of the basal diet.

Growth on the 50-percent bentonite diet is only partially restored when choline or additional protein are added to the diet, but the fatty livers do not appear. Methionine has no preventive or corrective effect.

It is concluded that the bentonite, as a cation-exchange silicate, removes choline from the intestinal contents thus producing the deficiency, but that in all probability other materials may be removed so that the deficiency is not a simple one.

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Hepatomas in Mice on a Diet Containing Bentonite [1,2]

J. WALTER WILSON,[3] *Arnold Biological Laboratory, Brown University, Providence, R. I.*

Mice fed an adequate semisynthetic diet mixed with an equal weight of bentonite failed to grow normally and developed fatty livers. Further experiments indicated that this was due to a deficiency of choline and possibly of other factors, removed from the intestinal contents by bentonite due to its base-exchange capacity (*1*). Such mice maintained for long periods developed hepatomas. This is consistent with the experience of Engel *et al.* (*2*) with rats on choline-deficient diets and with our own experience with mice on diets low in protein and deficient in choline (*3*). It is the purpose of this paper to discuss the occurrence and development of these hepatomas.

Materials and Methods

The mice used were strains BUA, BUB, and BUC, maintained in this laboratory for use in a program of investigation of the histophysiology and histopathology of the mouse liver. They have been inbred for over twenty generations and selected for excellence of growth and reproduction. They are maintained in an air-conditioned room at about 25° C. and routinely fed Purina laboratory chow.

The basal diet used is as follows: casein (Borden's "Edible") 20%, cornstarch (Argo) 40%, Crisco 20%, corn oil (Mazola) 4%, salt mixture (GBI-USP No. 2) 6%, yeast (GBI) 8%, and cod-liver oil (Squibb) 2%. The bentonite is obtained from the American Colloid Co. as "Volclay Bentonite." The low-protein, choline-deficient diet is a modification of that of Lowry *et al.* (*4*) made up as follows: casein ("Vitamin Test" GBI) 4%, cornstarch (Argo) 86.5%, cod-liver oil (Squibb) 2%, Wesson oil 3%, cystine 0.5%, and vitamins as follows (per 1,000 gm. of diet): pyridoxine 5 mg., thiamine 5 mg., niacin 10 mg., Ca pantothenate 50 mg., riboflavin 10 mg. Mice on these diets are kept in cages with wire mesh bottoms and weighed at least once a week.

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[2] Supported in part by grants from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, and from the Rhode Island Cancer Society.
[3] With the technical assistance of Miss Mary Day, Mrs. Harriet Parkhurst, and Miss Carolyn Swaney.

In order to follow changes in the liver, some of the mice received intravenous injections of thorotrast before the experiment was begun (*5*). Young mice, 35 days of age, received three injections of 0.1 cc. of thorotrast intravenously, given on alternate days. X-ray pictures were taken at intervals, usually once a month.

At sacrifice, pieces of the liver were fixed in Bouin's fluid, sectioned at 5 μ, and stained in Delafield's hematoxylin and eosin. In some cases pieces of liver were also fixed in Rossman's fluid and stained for glycogen by the Bauer-Feulgen method. The liver was also fixed in 10-percent formalin and stained for fat with Sudan IV and for reticular fibers by Gomori's method.

Experimental Results

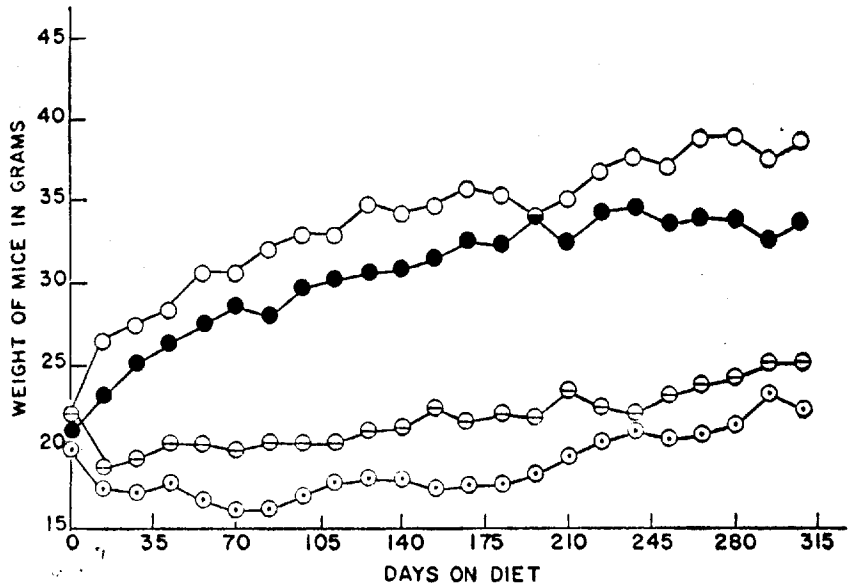
In all, 12 mice have been maintained on the 50/50 bentonite-basal diet for periods long enough to develop hepatomas (over 200 days), and hepatomas were found in all but one. Eight of these had had thorotrast injections and X-ray pictures had been taken. Figures 1 and 2, taken on the 167th and 200th day on the diet, show the development of one hepatoma—pictured at sacrifice on the 203rd day in figure 3. The length of treatment and incidence of tumors are given in table 1; the growth curves for one experiment are given in text-figure 1.

TABLE 1.—*Tumor incidence in mice fed the various diets, and controls*

Experimental treatment	Strain	No.	Period of treatment (*days*)	No. with tumors
50/50 Bentonite-basal diet	BUC	10	240–480	9
50/50 Bentonite-basal diet	BUB	2	203–269	2
Controls, basal diet, 51 A	BUC BUB	7 2	211–375 283–294	1 0
50/50 Ruffex-basal diet	BUC	9	280+	0
Thorotrast injections	BUC (varied)	18	216–571	0
Low-protein, choline-deficient diet	BUC BUA	7 2	232–437 444	2 1
BUC mice from stock	BUC	24	484–575	0

The occurrence of a hepatoma in one control animal that had been fed on the basal diet, and had been injected with thorotrast, necessitated a survey of strain BUC mice for spontaneous hepatomas. In 24 mice taken from stock, ranging from 484 to 575 days of age, there were no hepatomas. Since all our strains of mice have been developed for and extensively used in liver studies, we have been on the lookout for spontaneous hepatomas and until the present experiments have found only one in animals not on diets planned for the production of hepatomas.

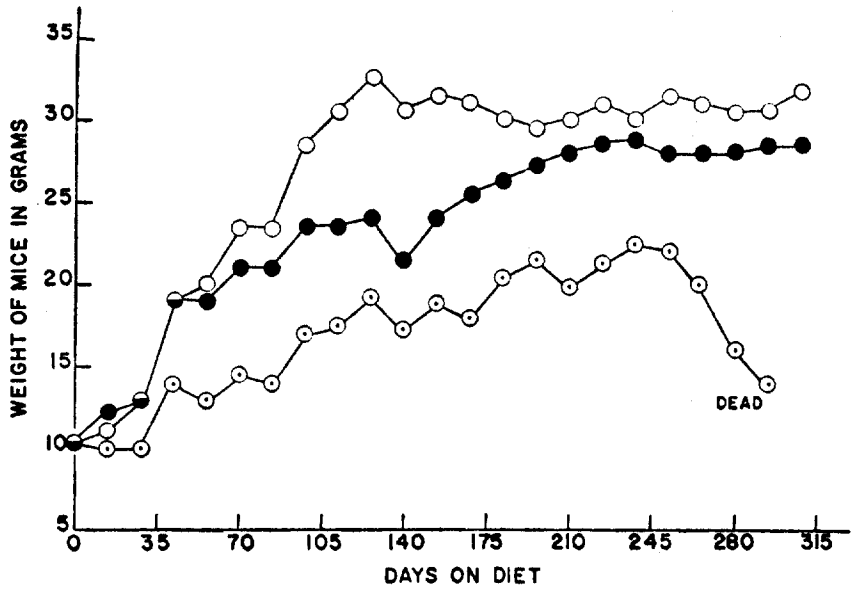
the one case was in strain BUA. In the present experiments only one hepatoma has been found in nine control mice. The alternate possibility, that the hepatoma in this one control might have been caused by the injected thorotrast, was also considered because of the fact that the thorium dioxide is radioactive. However, in 18 animals (not in these experiments) that had lived for 216 to 511 days after the injection of thorotrast, no hepatomas have been found.



TEXT-FIGURE 1.—Weights of strain BUC mice as follows: ○—male on basal diet; ●—female on basal diet; ⊖—Average of three males on 50/50 bentonite-basal diet; ⊙—average of three females on 50/50 bentonite-basal diet. All mice had received 3 injections of 0.1 cc. of thorotrast intravenously and were placed on the diets at 35 days of age. All mice on the 50/50 bentonite-basal diet, with the exception of one male, developed hepatomas.

The production of hepatomas in mice by feeding a diet low in protein and deficient in choline has already been reported (*3*). These experiments were conducted primarily to study the histologic and cytologic changes during the development of cirrhosis. Animals were placed on the diet at weaning, were sacrificed regularly, and only a few lived to the period when hepatomas might be expected. Furthermore, the diet used—a modification of that used by Lowry *et al.* (*4*) for the production of cirrhosis in rats—is not favorable for long survival. After varying periods on the diet, mice became anemic and suddenly died. On the other hand, a few continued to live for long periods with no apparent difficulty except slow growth and small size. In one experiment two mice were sacrificed after 437 days, and in another two were sacrificed after 444 days. The livers of all the mice that survived the first few weeks showed considerable fibrosis, loss of normal architecture, and co

tained ceroid. Of nine mice that survived more than 200 days, three had hepatomas. The growth of the mice fed this diet is shown in text-figure 2.



TEXT-FIGURE 2.—Weights of three strain BUA mice placed on low-protein, choline-deficient diet at 19 days of age. ●—♀ 31991 had a hepatoma when sacrificed at 444 days of age.

When 50% Ruffex replaced the 50% bentonite of the 50/50 bentonite-basal diet, the mice showed satisfactory growth and no hepatomas developed (table 1). In one experiment the daily food intake per mouse on the following diets was determined: basal, 3.37 gm.; 50/50 bentonite-basal, 6.14 gm.; and 50/50 Ruffex-basal, 5.92 gm. Since half the dietary intake on the last two diets was not food, the actual food intake was as follows: basal, 3.37 gm.; 50% bentonite, 3.07 gm.; and 50% Ruffex, 2.96 gm. Thus, slight inanition may have resulted from both the diets containing 50% bulk-former. This may account for the growth rate of the animals fed the 50/50 Ruffex-basal diet being slightly below the controls, but would not explain the extreme retardation of growth of the animals fed the 50/50 bentonite-basal diet.

Discussion

The original intention of the experiments in which a large amount of bulk-former is included in the diet was to produce sustained inanition with the hope of extending previous studies (*6*). The decreased food intake and decreased growth rate of the mice fed the 50/50 Ruffex-basal diet were only slight, and were accompanied by no discernible cytologic

changes in the liver. The extreme retardation on the 50/50 bentonite-basal diet, on the other hand, was accompanied by the development of fatty livers and eventually of considerable fibrosis and ceroid, and ultimately of hepatomas. The decrease in food intake, at least during the initial period of the experiment, was not more pronounced than on the 50/50 Ruffex-basal diet. The cytologic changes must then be due to some factor other than inanition, and we have shown that apparently choline and probably other dietary deficiencies may be involved (*1*). Furthermore, from the work of Tannenbaum and Silverstone (*7*), it is clear that moderate inanition, rather than producing hepatomas in mice, delays the development and decreases the number of spontaneous hepatomas and azo-dye-induced hepatomas.

The production of hepatomas in rats by feeding diets low in choline and available methyl groups has been extensively studied by Engel and his coworkers (*2*). We have reported the occurrence of hepatomas in mice on diets low in protein and deficient in choline (*3*). This has been further corroborated, with the same diet, in the present study. The addition of as little as 10 percent bentonite to the low-protein, choline-deficient diet prevents growth entirely (*1*). The addition of choline to the 50/50 bentonite-basal diet (*1*) prevents the fatty livers usually produced on this diet and permits better although not normal growth. The evidence, then, indicates that choline is removed from the contents of the intestine by bentonite and that a large amount of bentonite in the diet produces a deficiency in which choline is involved. This may account, in part at least, for the development of hepatomas on the 50/50 bentonite-basal diet.

Since the addition of choline does not restore growth to a normal level, it would appear that the deficiency is not a simple choline deficiency. Other cationic materials may also be removed by bentonite, thus contributing to the deficiency. This may be of importance in the development of the hepatomas and may account for the fact that they appear with greater frequency with the 50/50 bentonite-basal diet than with the diet low in protein and deficient in choline. The nature of these materials and their role in tumor production are being further investigated.

The occurrence of one hepatoma in a control animal is worthy of special comment inasmuch as this is unique in our experience with mice of this strain (BUC), although a large number of them have been used in long-term experiments. Silverstone *et al.* (*8*) have recently reported an increased frequency of spontaneous hepatomas in C3H mice on synthetic diets compared with those on natural foods. Our control was on a semi-synthetic diet, the "basal" diet of the bentonite experiments, but many other animals on this and on other similar diets have not developed hepatomas, which makes this explanation extremely doubtful. The presence of thorotrast is another factor to be considered. The mouse had lived for over 12 months with the Kupffer cells laden with thorotrast. Thorotrast is radioactive and its use in roentgenography has accordingly been questioned. The only report of carcinogenic action in the liver,

however, is a human case reported by MacMahon *et al.* (*9*) which was not a hepatic-cell tumor but was described as a primary endothelial-cell sarcoma. Furthermore, many mice in our experiments have survived as long or longer than this one without developing hepatomas. Finally, the hepatoma in this case occurred in a liver that showed no other signs of damage (fig. 6), as was also true of the spontaneous hepatomas in C3H mice, whereas the hepatomas in the animals fed the 50/50 bentonite-basal diet were in livers otherwise severely damaged (fig. 5), with considerable fibrosis, ceroid, and loss of normal architecture. We conclude, therefore, that this is a spontaneous hepatoma only fortuitously associated with these experiments.

Summary

Hepatomas developed in 11 of 12 mice fed a semisynthetic diet mixed with an equal volume of bentonite. One tumor developed in 1 of 9 control mice, none in 9 mice ingesting a 50/50 mixture of the basal diet and Ruffex. No tumors developed in 18 mice injected with thorotrast, none in 24 control mice raised on the stock diet, but 3 of 9 mice ingesting a low-protein, choline-deficient diet developed hepatomas.

The livers of mice on the 50/50 bentonite-basal diet were severely damaged, as indicated by considerable fibrosis, ceroid, and loss of normal architecture. The liver damage noted in the group ingesting bentonite is consistent with that expected during prolonged choline deficiency. The removal of choline from the intestinal tract by the bentonite, a base-exchange silicate, is tentatively advanced as a partial explanation for the development of the hepatomas in the mice in these experiments.

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HEPATOMAS PRODUCED IN MICE BY FEEDING BENTONITE IN THE DIET

By J. Walter Wilson
Department of Biology, Brown University, Providence, R. I.

It is the purpose of this paper to report the occurrence of hepatomas in mice fed a complete semisynthetic diet mixed with an equal amount of bentonite by weight. The details of the experiments and the evidence that has led to the tentative conclusion that the hepatomas are the result, in part, of an induced choline deficiency are to be reported in detail elsewhere.[1,2] Bentonite is a cation exchange silicate and apparently removes choline from the contents of the digestive tract so that it cannot be absorbed into the body, thus resulting in a deficiency.

Mice can adapt themselves to eating a diet containing half its weight of such inert material as Ruffex or barium sulphate with only a slight decrease in growth rate (FIGURE 1). Where bentonite makes up 25 per cent of the diet, growth is also nearly normal; but with 50 per cent bentonite in the diet, growth is almost completely suppressed (FIGURE 2). The mice continue to live, however, and grow very gradually for several months, but do not reach a normal size. Since the mice eat as much food on the 50/50 Bentonite diet as on the 50/50 Ruffex diet, failure to grow is not due to inanition alone.

Histological studies reveal that after the first three weeks, there is more or less fat in the livers (PLATE 1; 1), apparently depending on whether or not the mouse is gaining weight. This is in keeping with the general observation that, even on a diet free of lipotropic substances, the liver may not contain fat if the animal is not growing. The fat appears first as fine droplets, but soon many of the liver cells are distended with single large drops. Eventually, after three or four months, there is some fibrosis with progressive loss of architecture (PLATE 1; 2), although typical "hob-nail" cirrhosis has not been seen. There is also the accumulation of masses containing a brownish pigment, apparently ceroid (PLATE 1; 3).

Hepatomas have been found in all but one of twelve mice that in three experiments have been kept over 200 days on the diet. These are typical benign hepatomas of mice, much like those produced by repeated CCl_4 treatment or occurring spontaneously in C_3H mice.

The fatty livers, fibrosis, ceroid, and hepatomas present a picture much like that we have previously reported in mice maintained for several months on a diet low in protein and deficient in choline.[3] The symptoms develop more gradually and the mice maintain a more thrifty appearance. On a choline-deficient diet, at sporadic intervals, individual mice suddenly become anemic and die, so that only a few have been kept on it long enough to develop hepatomas. It seems possible that, in these sudden deaths, toxic materials produced by the intestinal flora may be involved. If so, they may be removed by the bentonite, which might account for the more thrifty condition of animals on the 50/50 bentonite diet.

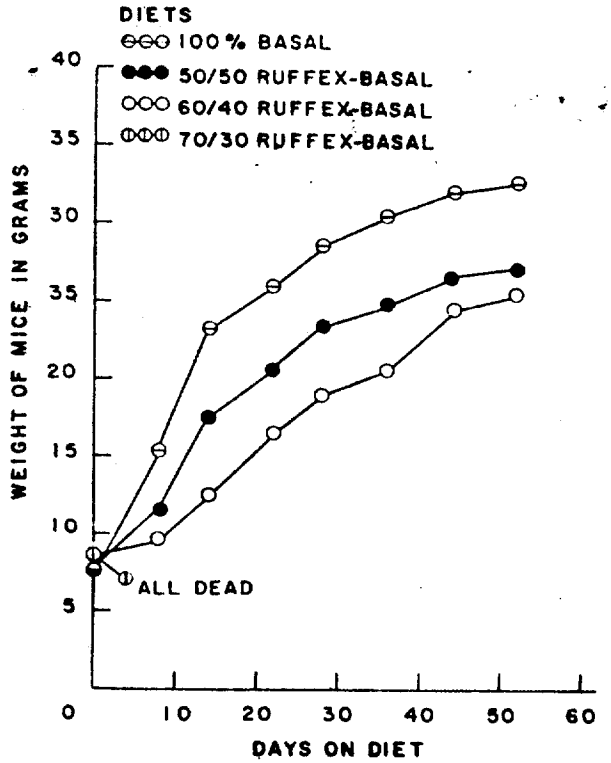

FIGURE 1

We have found that added choline will prevent the development of the fatty livers on the 50/50 bentonite diet, but that normal growth is only partially restored (FIGURE 2). When the amount of casein is doubled at the expense of corn starch, growth is also partially restored and the livers do not become fatty (FIGURE 3). This would be true of choline deficiency in both cases. When 1 per cent dl-methionine is added to the 50/50 bentonite-basal diet, however, the mice develop fatty livers and fail to grow (FIGURE 4). As a tentative explanation of this anomalous result, we suggest that the bentonite removes dibasic amino acids, and that the added methionine precipitates an incipient amino acid imbalance. These mice do not survive for long periods, the longest about 10 weeks, and with little gain in weight. It is only by nursing them along in a warm chamber at 31°C. that they can be maintained for any length of time at all. They have also developed a scaly tip to the tail, somewhat like that described for rats deficient in essential amino acids, which we have not as yet been able to cure.

It seems, then, that the deficiency is not a simple choline deficiency but may involve other essential basic materials removed from the intestinal contents by bentonite. It would be expected that some of the larger basic molecules would be taken up and held more firmly than inorganic cations, for with

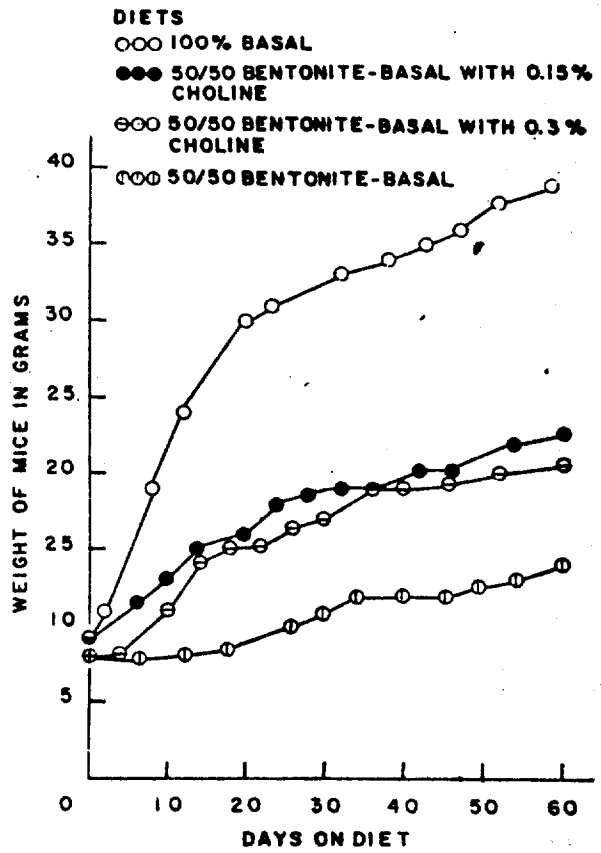

FIGURE 2

them, in addition to the Coulomb forces, Van der Waals forces come into play, and bases so held are only with difficulty eluted.[4] It would be difficult to predict what would be taken up under such complicated conditions as occur in the passage of the digestive tract. Experiments designed to test various possibilities are under way.

Except for the appearance of fat, there are no striking histological changes for the first four weeks. By the 60th day, there is a pronounced increase in the size of the nuclei, particularly in the centrolobular area (PLATE 1; 1–2). The cells are very much enlarged and the cytoplasm homogeneously finely granular and eosinophilic. The basophilia, usually conspicuous in the cytoplasm in this region, is absent and there are no glycogen vacuoles. Particularly in the animals with methionine added to the diet, there are no fat vacuoles because any mouse that has survived this long is no longer growing at all. Many of the enlarged nuclei contain conspicuous eosinophilic inclusions (PLATE 1; 4). Similar large cells with large nuclei and nuclear inclusions occur in the livers of

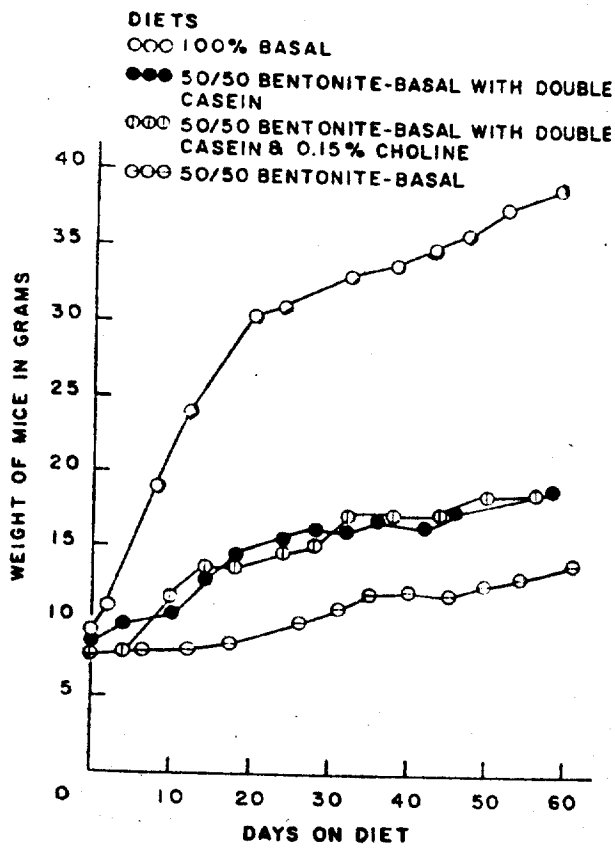

FIGURE 3

mice when they have been fed a diet containing a carcinogenic azo dye for a few weeks.[5]

The livers in which these enlarged cells appear are, in fact, very small. Apparently, there has been a gradual loss of cells with attempted replacement by cellular enlargement rather than by cell division. There are occasional mitotic figures throughout the series, but they are never abundant. When mice which have been 60 days on the 50/50 bentonite-basal diet and which have these enlarged liver cells are placed on a normal diet, rapid growth resumes and a spectacular wave of mitosis occurs in the liver which involves the large cells as well as the small ones (PLATE 2; 3). The large nuclei are obviously polyploid, for they have very wide-angled spindles and very large numbers of chromosomes. The metaphase illustrated in PLATE 2; 3 has a few chromosomes that have failed to reach the equatorial plate. From their size, the numbers on the plate must be great. The normal number for the mouse is 40.

Many of the figures are abnormal, with chromatin bridges persisting at anaphase and telephase (PLATE 2; 4). We have described abnormal figures of

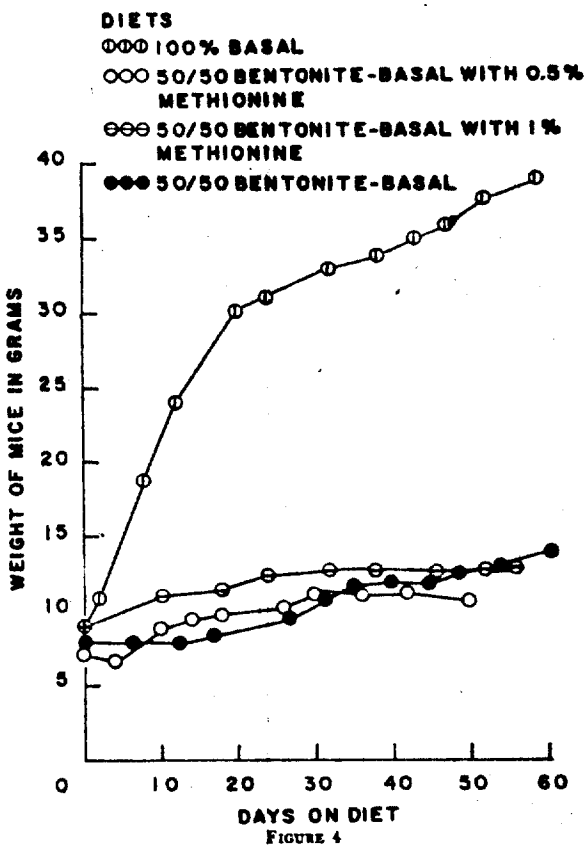

FIGURE 4

this sort in other cases where mitosis occurs in the mouse liver.[6] They occur also when the liver, regenerating after partial hepatectomy, is irradiated. Whether they result in viable cells with abnormal chromosome complements and thereby play a role in carcinogenesis as Brues suggests,[7] is an interesting speculation, and is worthy of further investigation particularly in light of the observation of Hauschka and Levan[8] on the occurrence of abnormal chromosome numbers in ascites tumors of mice.

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