Monograph on **Sulfates** D29 2/5/73

D29

SULFATES

A Monograph

Prepared for

Department of Health, Education and Welfare

Public Health Service

Food and Drug Administration

Rockville, Maryland

by

Dr. William Goldman

FIRL Project No. 80G-C3378-01

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SUMMARY

Introduction

The innocuous nature of sulfate ion is likely related to the rapid elimination from the body without any appreciable incorporation into metabolic pathways. Toxicity data is limited, probably mediated by the fact that large doses are required to yield untoward effects.

Acute Toxicity

Subcutaneous sodium sulfate in rabbits (175 mg/kg) led to "collapse" and death in 2 days, without urinary or fecal dysfunction (59). Subcutaneous doses of ammonium sulfate below ca. 600 mg/kg elicited hyperpnea and increased heart rate in rabbits; s.c. doses above 600 mg/kg led to increasingly severe cardiorespiratory discomfort, tetanic seizure and death in 4 hr (382). Cows drenched p.o. with ammonium sulfate (ca. 300 - 400 mg/kg) presented gastric upset and bloody diarrhea, superficial ecchymoses and petechiae in the thoracic and abdominal cavities, with death in 10 - 12 hr (382). The blood was abnormally fluid with retarded coagulation time. I.v. administration of 3 - 4 mg/kg ammonium sulfate in a human subject elicited behavioral disorder, incipient syncope and evidence of thrombosis (109).

Pharmacology

Isotonic solutions of sodium sulfate placed intraluminally exhibited an ability to induce a two-fold increase in gastrointestinal activity in dogs as compared to the effects of isotonic sodium chloride. Because the toxicity of the tested solutions are equivalent, it is suggested that this effect is anionically induced (408). Similar studies in the rat, rabbit, mouse and monkey (207) showed that isotonic solutions of sodium sulfate had no effect on the activity of isolated or intact intestines of these species and increased gastrointestinal activity and liquidity of content were both found to be correlated to the osmotic activity of the unabsorbed salt (207).

Fate studies

The absorption of orally administered Na_2SO_4 in rats initially occurs relatively slowly, rapidly increasing in rate with time. In these animals 56 and 70% of the label from a 1.4 and 2.8 g/kg oral dose, respectively, remained in the gut 4 hours after treatment, while 82% of the dose was excreted in the urine within 24 hrs (207). Sodium sulfate absorption from various sites, as with other similarly water-soluble salts, was dependent

upon a rich blood supply (513) and was diffusion controlled at isotonic or hypertonic concentrations (2, 485).

In general, very low levels of sulfate were retained within the body with only 6.5% remaining 100 min. after an intravenous dose of $Na_2S^{35}O_4$ in dogs (S2), divided between the trachea (0.6%), lungs (0.2%), liver (4.6%), spleen (0.26%), kidneys (0.3%) and brain (0.6%). In rats and rabbits the greatest levels were observed to be taken up by tissues containing high sulfomucopolysaccharides, such as gastrointestinal epithelium, cornea, and cartilagenous tissues (339) with little sulfate being incorporated into sulfur-containing amino acids (50). The normal gut flora is apparently required for the small amount incorporated into these amino acids (204). Also $Na_2SO_4^{18}$ given i.v. to rabbits retained most of the label in sulfate collected in the urine, indicating no oxygen interchange (26). Urinary excretion accounts for 67 - 84% of the dose in rats and rabbits (26, 107), within 24 hrs; an additional 10 - 14% is accounted for in the feces (118), the remainder being excreted more slowly or retained in body stores (339).

Effects on enzymes

A variety of nonspecific effects upon various enzyme systems result from sulfate treatment. RNA polymerase in the presence of 5% ammonium sulfate (506), liver glycerate dehydrogenase with 0.028 M sodium or ammonium sulfate (508), carboxylase-cocarboxylase with 0.16 mg/ml sodium sulfate (475) and pancreatic ribonuclease A with greater than 1 M concentrations of ammonium or sodium sulfate (509) were all found to be activated in the presence of the sulfate salt while diphosphoglycerate phosphatase with 8 mmolar sulfate (S4) and pancreatic ribonuclease A with less than 1 M concentrations of ammonium or sodium sulfate were found to be inhibited.

Physiology

The intravenous administration of isotonic solutions of sodium sulfate in human subjects produced an increase in urinary acid, potassium and ammonium output and a decrease in pH and chloride output without any appreciable change in urine flow (428). The role of the sulfate anion in establishing this result was not well defined and may not play a significant role in acid-base balance. It was suggested that independent mechanisms such as tubular thresholds of ionic reabsorption govern the excretion of

fixed ions (463). In contrast, sulfuric acid-induced metabolic acidosis resulting in increased urine flow with concomitant increases in urinary sodium excretion relative to other similarly administered acids results in the conclusion that this effect is attributable to anionic effects upon renal tubular reabsorption and permeability (58).

Alteration of membrane permeability by sulfate salts has been inferred in other studies. Intravenous sodium sulfate at doses less than 0.04 g/100 ml of blood increases canine bilary volume and salt excretion (80); lymph flow is remarkably increased in the presence of an intravenous injection into toads of a 5% sodium sulfate solution at 1 ml/100 g body weight (515); and rat liver mitochondria lose potassium at an accelerated rate when incubated with 2.5 to 10 mM ammonium sulfate (481).

The inclusion of sulfate salts in varying amounts to basal diets evokes a considerable variation in physiologic parameters which appear to be species dependent. Weight gain was observed in the chick at sodium sulfate levels up to 0.5% with optimal effects occurring at 0.1% (407). No effect was observed in the rat when levels of sodium sulfate up to 2% were included in the diet for 3 1/2 to 4 weeks (311). On the other hand, sodium or ammonium sulfate included in the diet of sheep in amounts equivalent to 1% sulfur for 12 days, or as sulfuric acid, (269,270) caused a decrease in food intake and a concomitant decrease in weight gain. As much as 1.3% ammonium sulfate and 0.4% sulfuric acid in the diet have been given to human subjects without significant ill effects (203).

CHEMICAL INFORMATION

SULFURIC ACID

I. Nomenclature

A. Common names: oil of vitriol, sulfuric acid

B. Chemical name: sulfuric acid

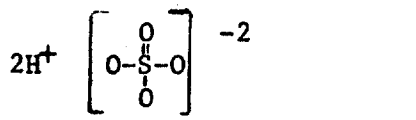
C. Trade name: none

D. CAS Reg. No. 7664939

II. Empirical Formula

H_2SO_4

III. Structural Formula



IV. Molecular Weight

98.07

V. Specifications

A. Food Chemicals Codex

limits of impurities

arsenic (as As) - 3 ppm

chloride - 0.2 ppm

heavy metals (as Pb) - 20 ppm

iron - 200 ppm

lead - 5 ppm

nitrate - 10 ppm

reducing substances (as SO_2) 40 ppm

selenium 20 ppm

B. Chemical Grade

1. Fisher Chemical Co.

assay - 95% min

limits of impurities

heavy metals (as Pb) - 0.8 ppm

iron - 0.2 ppm

residue after ignition - 4 ppm

arsenic - 0.0 ppm

chloride 0.2 ppm

nitrate - 0.5 ppm

ammonia - 1 ppm

subs. reducing $KMnO_4$ (as SO_2) - 1 ppm

2. J. T. Baker Chemical Co.

assay - 95% min

color (APHA) - 10

limits of impurities

residue after ignition - 4 ppm

chloride - 0.1 ppm

nitrate - 0.5 ppm

ammonia - 1 ppm

arsenic - 0.01 ppm

copper - 0.5 ppm

heavy metals (as Pb) - 1 ppm

iron - 0.2 ppm

nickel - 0.5 ppm

substances reducing permanganate - 1 ppm

3. M C & B Manufacturing Chemists

same as J. T. Baker Chemical Co.

VI. Description

A. General: clear, colorless, oily liquid, caustic and corrosive (Food Chemicals Codex).

B. Physical properties

m.p. (98.3%) 3.0

b.p.- ca. 338°

solubility

miscible with water and with alcohol

C. Stability

stable in air

AMMONIUM SULFATE

I. Nomenclature

A. Common names: ammonium sulfate

B. Chemical name: ammonium sulfate

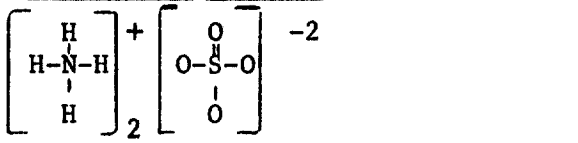
C. Trade name: none

D. CAS Reg. No. 7783202

II. Empirical Formula

$(NH_4)_2HSO_4$

III. Structural Formula



IV. Molecular Weight

132.14

V. Specifications

A. Food Chemicals Codex

limits of impurities

arsenic (as As) - 0.2 ppm

heavy metals (as Pb) - 10 ppm

residue on ignition 0.25%

selenium 30 ppm

B. Chemical Grade

1. Fisher Chemical Co.

limit of impurities

insolubles - 50 ppm

residue after ignition - 50 ppm

chloride - 5 ppm

heavy metals - 5 ppm

nitrate - 10 ppm

phosphate - 5 ppm

arsenic - 0.2 ppm

iron - 5 ppm

pH of a 5% sol/n. at 25°C - 5.0 - 6.0

2. J. T. Baker Chemical Co.

same as Fisher Chemical Co. except

chloride - 3 ppm

phosphate - 3 ppm

heavy metals - 3 ppm

iron - 2 ppm

3. M C & B Manufacturing Chemists

same as Fisher Chemical Co.

VI. Description

A. General: colorless or white, odorless crystals or granules (Food Chemicals Codex)

B. Physical properties

m.p. 235°

b.p. - not available

solubility

H_2O - 1g/1.5 ml insoluble in alcohol

C. Stability

stable in air, decomposes at temperatures above 280°C

CALCIUM SULFATE

I. Nomenclature

A. Common names: calcium sulfate, plaster of Paris, gypsum ($CaSO_4 \cdot H_2O$)

B. Chemical name: calcium sulfate

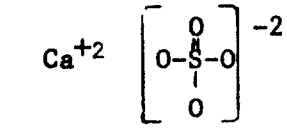
C. Trade name: none

D. CAS Reg. No. 7778189

II. Empirical Formula

$CaSO_4$

III. Structural Formula



IV. Molecular Weight

136.14

V. Specifications

A. Food Chemicals Codex

limits of impurities

arsenic (as As) - 3 ppm

fluoride - 30 ppm

heavy metals (as Pb) - 10 ppm

selenium - 30 ppm

B. Chemical Grade

1. Fisher Chemical Co.

limits of impurities

insoluble in HCl - 500 ppm

organic substances - to pass test
acidity (as H_2SO_4) - 100 ppm
carbonate - 300 ppm
chloride - 50 ppm
nitrate - 50 ppm
alkalies and magnesium - 2000 ppm
heavy metals (as Pb) - 20 ppm
iron - 20 ppm

2. J. T. Baker Chemical Co.
   assay - 99.0 - 102.0%
   insoluble in HCl - 500 ppm
   chloride - 20 ppm
   nitrogen compounds (as N) - 20 ppm
   carbonate (CO_3) - 300 ppm
   heavy metals (as Pb) - 20 ppm
   iron - 20 ppm
   magnesium and alkalies (as SO_4) - 2000 ppm
3. M C & B Manufacturing Chemists
   alkalies and Mg salts - 2000 ppm
   chloride - 20 ppm
   heavy metals (as Pb) - 20 ppm
   insoluble matter - 500 ppm
   insoluble in HCl - 500 ppm
   iron - 20 ppm
   nitrate - 50 ppm

VI. <u>Description</u>

A. General: fine, white to yellow-white, odorless powder (Food Chemicals Codex)

B. Physical properties
   m.p. (anhydrous) 1450°
   b.p. - not available
   solubility
   H_2O - 2gm/l at 19°C

C. Stability
   stable in air

POTASSIUM SULFATE

I. Nomenclature

A. Common names: potassium sulfate

B. Chemical name: potassium sulfate

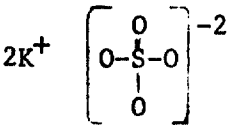
C. Trade name: none

D. CAS Reg. No. 7778805

II. Empirical Formula

K_2SO_4

III. Structural Formula



IV. Molecular Weight

174.26

V. Specifications

A. Food Chemicals Codex

limits of impurities

arsenic (as As) - 3 ppm

heavy metals (as Pb) - 10 ppm

selenium - 30 ppm

B. Chemical Grade

1. Fisher Chemical Co.

limits of impurities

insolubles - 100 ppm

chloride - 10 ppm

arsenic - 0.5 ppm

nitrogen compounds (as N) - 5 ppm

sodium - 200 ppm

calcium, magnesium and R_2O_3 ppt - 200 ppm

heavy metals (as Pb) - 5 ppm

iron - 5 ppm

pH of a 5% sol'n at 25°C - 5.5 - 8.5

2. J. T. Baker Chemical Co.

same as Fisher Chemical Co.

3. M C & B Manufacturing Chemists

same as above except

arsenic - 2 ppm

VI. Description

A. General: colorless or white crystals or crystalline powder having a bitter, saline taste (Food Chemicals Codex)

B. Physical properties

m.p. 1067°

b.p. - not available

solubility

H_2O 1 gm/8.5 ml insoluble in alcohol

C. Stability

stable in air

SODIUM SULFATE

I. Nomenclature

A. Common names: sodium sulfate

Glauber's salt

B. Chemical name: sodium sulfate

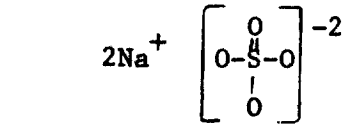
C. Trade name: none

D. CAS Reg. No. 7757826

II. Empirical Formula

Na_2SO_4

III. Structural Formula



IV. Molecular Weight

142.04

V. Specifications

A. Food Chemicals Codex

limits of impurities

arsenic (as As) - 3 ppm

heavy metals (as Pb) - 10 ppm

selenium - 30 ppm

B. Chemical Grade

1. Fisher Chemical Co.

limit of impurities

insolubles - 100 ppm

loss on ignition - 5000 ppm
chloride - 20 ppm
nitrogen compounds (as N) - 5 ppm
arsenic - 0.5 ppm
calcium, magnesium and R_2O_3 ppt - 200 ppm
heavy metals (as Pb) - 5 ppm
iron - 10 ppm
pH of a 5% sol'n. at 25°C - 5.2 - 8.2

2. J. T. Baker Chemical Co.
   same as Fisher Chemical Co. except
   chloride - 10 ppm
   calcium, magnesium and R_2O_3 ppt. - 100 ppm
   iron - 5 ppm
   potassium - 100 ppm
3. M C & B Manufacturing Chemists
   same as Fisher Chemical Co. except
   arsenic - 1 ppm

VI. <u>Description</u>

A. General: colorless crystals or as fine, white, crystalline powder (Food Chemicals Codex).

B. Physical properties
   m.p. 884
   b.p. --- not available
   solubility
   H_2O - freely soluble
   practically insoluble in alcohol

C. Stability
   The decahydrate is efflorescent

Analytical Methods

1. AOAC, 1970 pp. 142, 44

   In baking powders: Add dil. HCl to a weighed sample and boil for 1.5 hrs. Filter, wash and add an excess of 10% $BaCl_2$ dropwise to the boiling soln of filtrate plus wash. Cool, filter, wash and ignite the precipitate. Weigh as $BaSO_4$.

2. AOAC, 1970 pp. 186, 522

   In wines and vinegar: To 100 ml add ca. 1 N HCl, heat to boiling and add 10 ml 1% $BaCl_2$ dropwise. Filter on ashless filter paper. Wash, dry and ignite the precipitate to 700 - 800°. Cool it and weigh as $BaSO_4$.

3. AOAC, 1970 pp. 573

   In salt: Dissolve 20 gm in H_2O and add dilute HCl. Heat to boiling, filter and wash. To the combined filtrate plus wash, continue as in number 2, above.

Occurrence

A. Plants: Sulfate is present in small amounts in all plant life

B. Animals:

Human extracellular fluid	1mEq/1[a]
Human intracellular fluid	2mEq/1[a]
Human serum	0.5 - 1.5mEq/1[b]

C. Synthetics: none

D. Natural Sources: Sulfates are found naturally as crystalline salts. Typical examples are as follows[c]:

Anhydrite	$CaSO_4$
Barite	$BaSO_4$
Epsomite	$MgSO_4 \cdot 7H_2O$
Gypsum	$CaSO_4 \cdot 2H_2O$

E. Uses as food additives

(1) Dough conditioner

(2) Nutrient, Dietary supplement

(3) Yeast food (bread, malt liquor, wine)

(4) Firming agent (salt, garlic and onion salt, canned vegetables)

(5) Sequestering agent (ice cream, preserved meat and sea foods)

(6) Water conditioner

(7) Acidifier

a. Guyton, A. C. *Textbook of Medical Physiology*. W. B. Saunders co., 1971.

b. Harper, H. A. *Review of Physiological Chemistry*. 9th edition. Lange Medical Publishers, 1963.

c. Kraus, E. H., W. F. Hunt and L. S. Ramsdell. *Mineralogy*. 5th edition, McGraw Hill Book Company Inc., 1959.

BIOLOGICAL DATA

I. Acute toxicity

1. Ref. 382 ($(NH_4)_2SO_4$)

animal	species	sex/no.	route	dose	measurement
A	rabbit	?/1	s.c.	1 gm/kg	LD
B	"	"	"	660 mg/kg	LD
C	"	"	"	500 mg/kg	Sub-LD
D	"	"	"	400 mg/kg	Sub-LD

Animals A and B showed the same morbid signs. Within 10 min after injection they began breathing very rapidly and through the mouth; heart beat quickened and became violent. The animals suffered occasional violent tetanic convulsions and then became more and more quiescent until death ensued ca. 4 hrs later. Animals C and D showed the same but less severe symptoms (except no tetanic convulsions in C) and recovered within ca. 2 hrs. Blood sampled from A and C was very fluid and coagulated slowly (ca. 1 hr instead of the normal 10 min.). The minimum lethal dose was estimated at ca. 500-600 mg/kg.

species	sex/no.	route	dose	measurement
cow	f/1	p.o.	430 mg/kg	LD
"	steer/1	p.o.	290 mg/kg	LD

The cows suffered violent stomach aches and abundant and bloody diarrhea. Hemorrhagic purpura was observed on various parts of the body and lesions were found in the thoracic cavity and abdomen. Death ensued at 10 - 12 hrs. Blood samples were extremely fluid and coagulated very slowly. Inhibition of coagulation was confirmed *in vitro*.

2. Ref. 109 (ammonium sulfate)

species	sex/no.	route	dose	measurement
man	?/1	i.v.	3 - 4 mg/kg	ED*

*Effective dose

Administration of 1 ml of 20% ammonium sulphate induced nervous symptoms (incipient black-out) and thrombosis.

3. Ref. 59 (sodium sulfate)

species	sex/no.	route	dose	measurement
rabbit	?/1	s.c.	175 mg/kg	LD_{100}

The animal was said to have experienced "some degree of collapse". Urine was passed normally and there was no diarrhea. Death occurred 2 days post-injection.

II. Short-term studies

1. Ref. 269 (sulfuric acid)

The addition of 80 ml 4N H_2SO_4 per kilogram grass pelleted diets fed ad lib to a group of 8 sheep for 20 days resulted in a food intake which was 70% of the control group and 80% of controls when combined in a 50:50 mixture with HCl. The reduced dietary intake was also reflected in reduced body weight gain. The authors state that both acids produced a similar degree of metabolic acidosis and that plasma sulfate was raised while chloride was not.

2. Ref. 270 (Na_2SO_4, $(NH_4)_2SO_4$, H_2SO_4)

Sodium sulfate or ammonium sulfate was fed to 5 Wethers sheep (1% sulfur in the dry matter) ad lib for 12 days followed by an 11 day restricted intake. This procedure was followed by feeding equivalent amounts of sulfuric acid to these same 5 sheep under an identical regimen.

Sodium sulfate reduced food intake 22% of controls while ammonium sulfate and sulfuric acid reduced intake to 44% of controls. Correlation of diet intake to blood chemistry indicated that the reduced intake, due to ammonium sulfate and sulfuric acid, was in general related to the degree of metabolic acidosis as reflected by decreased blood pH and a reduced pCO_2. Sodium sulfate, however, produced no appreciable change from controls levels in blood pH, pCO_2, water intake, urine volume, rumen content pH or fecal water content. Sodium sulfate on feed intake was suggested by the authors to be related to the high sulfate intake, since higher levels of sodium as sodium chloride, did not reduce feed intake of young lambs.

III. Long-term studies

No information available

IV. Special studies

1. Ref. 311 (sodium sulfate)

Weanling male Sprague-Dawley rats fed either 0.88, 8.64 or 138 mmole sodium sulfate per kg of basal diet for 3 1/2 to 4 weeks exhibited no significant differences in weight gain, feed intake, feed-gain ratio or water intake as compared to control rats. Hemoglobin, red blood cell count, white blood cell count, serum protein, alkaline phosphatase and inorganic phosphate values were similarly unaltered. Variations in gastrointestinal organ weights or length of small intestine or color were unaffected by the dietary regimen.

2. Ref. 317 (sodium sulfate)

Female guinea pigs received $Na_2{}^{35}SO_4$ (route?) at various stages of estrus and anestrus. The genital tracts were excised, stained and examined autoradiographically. Initially, sulfur label during estrus appeared weakly in the glandular cells, cylindrical cells, connective tissue and stratified epithelium of the vaginal neck. As estrus progressed the label concentrated within the apical poles of the cylindrical cells of the cervix. The concentration of sulfur-35 in the cervix rapidly diminished during anestrus, indicating a modification of the functional kinetics.

3. Ref. 407 (sodium sulfate)

A high energy corn-soy basal diet low in added sulfate and deficient in methionine was fed to broiler-type chicks. In three experiments graded levels of sodium sulfate with or without graded levels of methionine were fed for 7 or 8 weeks. There were consistent increases in weight with the addition of 0.05 to 0.5% of sodium sulfate to the basal diet with little additional significant weight gain at levels greater than 0.1%.

A dietary requirement for sulfur from inorganic sulfate apart from sulfur amino acids in the broiler is indicated by the results of this study.

4. Ref. 505 (sodium sulfate)

The addition of 0.5% indole to a basic diet caused an immediate cessation of growth in weanling female Wistar rats. Adding 0.25 or 1.0% sodium sulfate to this combination reinstituted growth for an 18 to 21 day period followed by a second stationary period. Replacing the sodium sulfate with cysteine or methionine caused a vigorous

recovery of growth by 12.5 and 14 g., respectively, within 9 to 12 days. Thus sodium sulfate, which is not capable of conjugating organic compounds like indole, does provide a conservative effect for the sulfur amino acids which are essential for the binding of indole.

BIOCHEMICAL ASPECTS

I. Breakdown

No information available

II. Absorption - distribution - metabolism - excretion

1. Ref. 26 (sodium sulfate)

Sodium-^{18}O-sulfate (1 g in 50 ml) was injected in a rabbit and the urine was collected for 24 hours. During this period 84% of the administered sulfate (corrected for control sulfate excretion) was excreted and retained most of the label. Thus, little exchange of administered sulfate with tissue sulfate occurred prior to excretion.

2. Ref. 50 (Na_2SO_4)

$Na_2{}^{35}SO_4$ administered i.p. to rats was shown to be incorporated in small amounts into chondroitin sulphuric acid isolated from the costal cartilage and in trace amounts into taurine isolated from the liver, with the maximum incorporation occurring within 24 and 8 hours, respectively. It was also shown that exogenous sulphate is not incorporated into methionine or cystine. The authors conclude that the main uptake of exogenous sulphate occurs in mucopolysaccharides containing ester sulphates.

3. Ref. 101 (sodium sulfate)

$Na_2{}^{35}SO_4$ was administered i.v. to rabbits, which were then sacrificed at various time intervals, the eyes enucleated, analyzed for radioactive sulfate content, submitted to autoradiographic studies and assayed for proportionate distributions of inorganic sulfate, ester-sulfate and organic nonsulfate sulfur.

Radioactive sulfur was rapidly taken up by the eye with the highest levels occurring in vascular tissue such as the uvea, sclera and vitreous body plus retina. Turnover rates were also high in these same tissues but slow in the cornea and lens.

Autoradiographic studies at 3 days after administration of the test compound showed high S^{35} content in the inner layers of the cornea and sclera with low levels occurring in the uvea, retina and the pia and dura of the optic nerve head and no radioactivity within the aqueous humor or vitreous body.

The sulfate was found to be incorporated principally into sulfomucopolysaccharides, especially in the cornea and sclera, while nonsulfate sulfur was found in large amounts in the lens with cystine and methionine accounting for 40% of the nonsulfate label in the cornea.

4. Ref. 107 (sodium sulfate)

$Na_2{}^{35}SO_4$ (1 mg in 1 ml dist. water) was given i.p. to fourteen male and thirteen female adult rats (180 - 330 gm). Animals were sacrificed at various intervals and samples of blood, liver, brain, bone and bone marrow were assayed for S^{35}.

Approximately 67% of the administered S^{35} was excreted in the urine within the first 24 hours and 85% within 120 hours. The addition of fecal excretion of S^{35} accounts for 95% of the administered dose within 120 hours.

Rapid elimination of sulfate was observed to occur from the blood, liver and brain with nearly complete elimination occurring within 48 hours. However, a pronounced rise in S^{35} concentration was found in bone and bone marrow with maximum concentrations occurring in 8 hours in bone and 24 hours in marrow. Elimination of S^{35} from bone and bone marrow is relatively slow with significant concentrations being present 120 hours after administration.

5. Ref. 118 (sodium sulfate)

Adult female rats with bile fistulas were given a single i.v. dose of 1 μg $Na_2{}^{35}SO_4$. The animals were sacrificed from 1 - 24 hours after administration and the amount of S^{35} excreted in urine, bile and feces were measured.

After 24 hours 89% of the total injected ^{35}S was excreted. Seventy-five percent of the sulfur was recovered in the urine, 10% in the bile, and 4% in the feces. Determinations of the sulfur content of segments of gastrointestinal tract of these rats showed that ^{35}S enters the upper third of the small intestine from blood or tissue fluid and is excreted in the feces.

6. Ref. 127 (sulfuric acid)

A 25 ml/kg dose of 0.1 N H_2SO_4 by gavage in earlier studies on fasted cats was 6% neutralized by ammonia combination within 24 h. A 100 ml/kg dose merely doubled the amount of ammonia produced during the first 24 hrs. Although ca. 100% of the extra sulfate was excreted

within that interval, only ca. 30% of it was bound to ammonia at either dose. However, the ammonia output continued to remain elevated, such that the 3 day total would have approximated 60% neutralization.

Fixed base excretion was somewhat higher after the 100 ml/kg than the 25 ml/kg acid dose. But this result does not suggest that the available ammonia has been exhausted, since less ammonia is initially generated when the acid load is high. Sodium and potassium are about equally elevated 24 hrs post treatment. This shows that a sufficiently large dose of acid will remove sodium even in fasted animals, and based on potassium distribution in the cat, fixed based is leached from sources other than circulating body fluids. Sodium and potassium retention returns more rapidly than normalization of ammonia excretion. It is not known whether fixed base was excreted because ammonia was not generated sufficiently rapidly. However, it is clear that "something more than an unessential surplus has been tapped", given the evidence for base retention during fasting.

7. Ref. 156 (sodium sulfate)

Sodium sulfate (0.5%) added to a basal diet did not improve growth in chickens. Chickens were found to retain initially 50 to 75% of oral doses of $Na_2{}^{35}SO_4$ and continued to retain 15 to 30% of the compound 10 to 14 days later. The retention occurred prominently in the mucopolysaccharide - mucoprotein sulfate fractions of connective tissue, and also in liver taurine. Small amounts of label incorporated into sulfur amino acids was thought to be due to bacterial action of the alimentary tract.

The authors conclude that the chicken satisfies part of its total sulfur requirement from inorganic sulfate, although the sulfate source can not substitute significantly for cysteine or methionine in protein synthesis.

8. Ref. 204 (sodium sulfate)

$Na_2{}^{35}SO_4$ was administered i.p. to 5 germ-free and 5 conventional Swedish strain rats. Blood, liver, kidneys, pancreas, stomach and the whole intestine with its contents were excised and analyzed 3 hours after injections for radioactive content. See the following table:

The percent of injected label in whole organs and total serum

Group	Serum	Liver	Kidneys	Pancreas	Stomach plus intestine
germ-free	1.40	3.80	0.74	0.07	17.7
conventional	1.17	4.62	0.80	0.11	13.2

Based on the distribution and intestinal excretion of the labeled compound no marked differences between germ-free or conventional animals were noted, although gastrointestinal tract label tended to be slightly higher in germ-free animals. Label in serum, liver, kidney and pancreas was incorporated into cysteine and methionine in conventional but not in germ free animals, indicating gut floral activity in the conversion.

9. Ref. 339 (sodium sulfate)

Tissues known to contain high sulfomucopolysaccharide levels such as gastrointestinal tract, cardiovascular tissue, liver, kidney and cornea were excised from five 200 g rats and from one adult rabbit previously administered $Na_2{}^{35}SO_4$ subcutaneously. A contrast-autoradiographic determination of the amount of S^{35} fixed within each tissue indicated a diffuse uptake of the radioactive sulfate with the greatest quantities being taken up by the epithelium and adventitial tunic of the esophagus, ileal epithelium, inner layer of the cornea and tracheal cartilage of the rat and cartilage plates and vascular tunica media of the rabbit lung. The direction of increasing label density in all tubular systems was toward the littoral layers.

10. Ref. 428 (sodium sulfate)

The infusion of one liter of 4% sodium sulfate to 6 normal human subjects resulted in a decrease of urinary pH from 6.05 to 4.32 with a doubling of ammonia and titratable acid excretion. In 10 patients with renal disease and normal serum bicarbonate (greater than 25.1 meq/l), an infusion of sodium sulfate resulted in a rise in urine pH without substantial change in ammonia or titratable acid excretion; thus, net acid excretion fell. In those same 10 patients with renal disease but with serum bicarbonate below 20 meq/l the infusion of sodium sulfate solution elicited a fall in urinary pH from 5.17 to 4.83 with a con-

comitant rise in ammonia and titratable acid excretion similar to that in normal subjects.

11. Ref. 485 (sodium sulfate)

The resorption of combinations of isotonic Na_2SO_4 and hexoses from the small intestine, ligated to equal lengths, was measured in dogs and rats. It was concluded that resorption of $SO_4^{=}$ is diffusion controlled.

12. Ref. 504 (sodium sulfate)

The comparative incorporation of labeled l-cystine-S^{35} and $Na_2{}^{35}SO_4$ by leukocytes *in vivo* was studied after p.o. injection to human patients, either with no hematologic abnormalities or acute leukemia, chronic myeloid leukemia or chronic lymphoid leukemia. Maximum levels of label appeared between days 5 and 12 in normal peripheral leukocytes and within 2 days in acute leukemia leukocytes. Sulfate label incorporation and duration was significantly less, quantitatively, than that from cystine label. Incorporation from sulfate label in chronic myeloid leukemia exceeded that for the acute disease but was still below normal leukocyte uptake. Incorporation in chronic lymphatic disease was vanishingly negligible. Leukemic plasma levels of sulfate label were similar for each condition and they disappeared more rapidly than after cystine label.

Label excretion in 24-hr. urine varied between 49 and 63% of initial dose for both acute and chronic states, while only 25% was eliminated in normal subjects during the same interval. Only chronic myeloid leukemic leukocytes incorporated significant sulfate label after 1 hr incubation in vitro, exclusive of the blast phase.

13. Ref. 512 (sodium sulfate)

Infusions of solutions of increasing concentrations of sodium sulfate in 5 dogs at a fast (4.19 ml/min.) or slow (0.45 ml/min) rate resulted in a steady state relationship between intake and output (isorrhea) at 3 hrs. The minimum isorrheic quantity for the salt was found to be 50 μeq/min, but a limiting isorrheic quantity could not be obtained. Minimum isorrheic concentrations of 50 meq/l were obtained for sodium and sulfate and limiting isorrheic concentrations of 420 and 480 meq/l were obtained for sodium and sulfate, respectively.

The rate of sodium or sulfate excretion per minute per unit load (velocity constant - α) was 0.0212 and 0.0317, respectively. These rates were more than 20X higher than the α Na and αCl when NaCl was infused. Since the diuretic behavior of sodium sulfate is approximately that of H_2O or isotonic glucose, the high sulfate excretion rate is attributed to its insignificant threshold of retention.

14. Ref. 513 (sodium sulfate)

Physiological saline (0.2 - 0.3 ml) containing $Na_2{}^{35}SO_4$ was injected into the pericardial cavity of puppies, cats, rabbits and rats and the label fate was followed by inverting type autoradiography. Sodium sulfate, like other water-soluble substances, was absorbed via the intercellular spaces between the capillary and venule endothelial cells after passing between the mesothelial cells in the serous epicardium or pericardium. Absorption from all areas of the mesothelium was uniform and independent of mesothelial cell size or type of reticular fiber (argyrophil, collagenous or elastic).

15. Ref. S2 (sodium sulfate)

$Na_2{}^{35}SO_4$ was injected into the femoral artery of a dog. Retention of the labeled sulfur after 100 min. was very low, with only 0.6% remaining in the trachea, 0.2% in the lungs with 4.6, 0.26, 0.3 and 0.6% retained by the liver, spleen, kidney and brain, respectively.

16. Ref. S3 (sodium sulfate)

Fasting female Wistar rats weighing ca. 260 g received sodium sulfate by gavage in a volume equivalent to 5% body weight. Assay of 4 - hour urine found ca. 7% of the sulfur load excreted as inorganic sulfate. Approximately 90% of the sulfur load in a volume of sodium sulfate equivalent to 3% body weight was found in 4 - hour urine after i.p. injection. These values confirm the very low tissue absorption of sulfate.

III. Effects on enzymes and other biochemical parameters

1. Ref. 30 ($(NH_4)_2SO_4$)

The addition of 0.1 ml of a saturated solution of $(NH_4)_2SO_4$ to 1 ml of an incubation medium containing liver nuclei RNA polymerase from adrenalectomized rats caused AMP-^{14}C incorporation into RNA to rise to 587 μμ moles per mg DNA from 252 μμ moles in sulfate free mixtures, after 15 min. Liver nuclei from rats pretreated with 50 mg cortisone/kg 4 hours before sacrifice exhibited slight increases in RNA polymerase activity. When cortisone pretreatment was combined with $(NH_4)_2SO_4$ a two-fold increase in activity was observed, suggesting that cortisone had little effect on RNA polymerase.

2. Ref. 58 (sulfuric acid)

High urinary osmotic levels of 1300 - 1970 m osm/l with low urinary flow of 0.15 to 0.30 ml/min were obtained in anesthetized female mongrel dogs deprived of water for 20 hours and food for 10 hours. The i.v. administration of 0.6M sulfuric acid at a rate of 0.2 to 0.5 ml/min with incremental increases of 0.5 ml/min every 30 to 40 min until rates of 3 to 5 ml/min were obtained resulted in an increase of urine flow of 2 - 4 ml/min. The urinary flow in terms of solute load were not found to be significantly different than dogs which were hydropenic alone. The relative diuretic effect of sulfate anion was similar to phosphate, while nitrate and chloride, which are reabsorbed in significant amounts, were less diuretic.

Determination of electrolyte excretion showed that urinary sodium excretion increased from 183 to 320 meq/l after sulfuric acid induced acidosis, while chloride excretion was rarely greater than 100 μeq/min (5.4 meq/l).

The authors concluded that acidotic diuresis was not influenced by serum pH but depended on tubular anion excretion mechanisms.

3. Ref. 59 (sodium sulfate)

The intravenous injection of sodium sulfate at 175 mg/kg into a single rabbit resulted in a serum calcium drop of 22% in 4 hours. Inorganic phosphorus was depressed by 34% at 1 3/4 hours, returning toward control levels at 4 hours. No change in serum magnesium was noted to occur.

4. Ref. 80 (sodium sulfate)

The intravenous injection of sodium sulfate at a dose less than 400 mg per liter of blood in a 15 kg dog increased biliary volume and biliary salt excretion two-fold. Increased bile salt production was thought to lower bile surface tension.

5. Ref. 104 (ammonium sulfate)

Concentrations of ammonium sulfate from 0.1 M to 0.25 M in an incubation mixture of "aggregate" RNA polymerase from rat brain nuclei before initiation of RNA synthesis resulted in a slight increase in the incorporation of [^{3}H] CTP into DNA. The enzyme activity was inhibited at salt concentrations above 0.41 M. Addition of 0.33 M ammonium sulfate to previously initiated RNA synthesis resulted in a 4x increase in enzyme activity. The addition of the enzyme to the incubation mixture containing ammonium sulfate increased the polymerase activity approximately 3x.

A comparison of polymerase activity from rat brain and liver revealed a biphasic effect of the ammonium sulfate on brain enzyme with two peaks occurring at 0.25 and 0.35 M, while liver nuclear enzyme activity exhibited only a single broad peak between 0.2 and 0.4 M.

Ammonium sulfate at an undisclosed concentration was also found to increase the incorporation of [^{3}H] UTP by "aggregate" RNA polymerase from rat brain nuclei in unfractionated, neuronal, and glial nuclei by 7, 10 and 6x, resp.

6. Ref. 108 (sodium sulfate)

Intravenous administration of isotonic sodium sulphate (0.17 molar) to anesthetized dogs at 5 ml/kg increased the salivary secretion from the submaxillary gland by approximately 20%, an effect similar to isotonic sodium chloride. While 5 ml/kg hypertonic sodium sulphate (1 molar) caused a decrease of 40% in the salivary secretion after an initial rise lasting about 2 minutes.

7. Ref. 109 (ammonium and sodium sulfates)

Oral ingestion of 20 g ammonium sulfate as enteric-coated capsules in man led to an increase in acid output (fall in urinary pH from 5.5 to 4.8) within a 3 hour period. A concomitant increase in the output of buffering substances, of which phosphate represents 50 to 70%, was also noted. The intravenous injection of sodium sulfate (200 ml of 20% concentration) produced an increase in urine acidity accompanied by a decrease in buffer output and an increase in ammonia output. A direct relationship between urine pH and ammonia output after the injection of hypertonic sodium sulfate was noted to occur.

8. Ref. 123 (sodium sulfate)

The administration of 2000 ppm sodium sulfate (75 - 130 ml/min) in an isolated kidney (species not stated) perfused by a heart-lung preparation led to the following effects: 1. a 5 - 10x increase in urine output, 2. increased sulfate excretion, 3. no significant change in renal blood flow, and 4. no significant change in renal oxygen consumption. The data suggest that sulfate diuresis is not associated with elevated renal oxygen consumption unless there is a concomitant increase in renal blood flow. This was confirmed by the results of 8 i.v. studies, where only a profuse sulfate diuresis associated with elevated renal circulation was generally accompanied by increased renal oxygen consumption.

9. Ref. 137 (ammonium sulfate)

$(NH_4)_2SO_4$ (1.25 mg in one ml containing 2 to 4 x 10^6 cells) completely suppressed hemagglutination-positive Col SK virus multiplication and corresponding cytopathic effect after a 3 - 4 day incubation with Ehrlich ascites cell cultures. A 0.63 mg/ml concentration of ammonium sulfate yielded a more than 75% inhibition of cytopathic effect with a hemagglutination titre of 320, while 0.31 mg/ml concentration led only to a 25% inhibition of cytopathic effect and a hemagglutination titre of 1280.

$(NH_4)_2SO_4$ was ineffective against the multiplication and infectivity of hemagglutination negative Col SK and LCM viruses.

10. Ref. 203 ($(NH_4)_2SO_4$, Na_2SO_4, H_2SO_4)

Gastric emptying time was studied in 6 fasting humans, given various solutes in test meals by stomach tube. The volume of test meal remaining after a fixed interval was then compared with the osmolar concentration of the solute, as a measure of gastric emptying.

Increasing concentrations of $(NH_4)_2SO_4$ (from 0 to 100 mmolar) and H_2SO_4 (0 to 30 mmolar) resulted in increasing gastric emptying times, an effect similar to most other solutes studied.

Low concentrations of Na_2SO_4 (ca. 15 mmolar) evoked rapid gastric emptying; higher concentrations, however, slowed this effect. It is suggested that sodium facilitates transport across the osmotic membrane at low concentrations. The action of acids is thought to be dependent on other mechanisms.

11. Ref. 207 (sodium sulfate)

A single oral administration of 1400 mg/kg $Na_2{}^{35}SO_4$ led to a mean 44 and 56% label recovery in urine (plus carcass) and feces (plus GI content), resp., after 4 and 8 hrs in 4 rats. A single p.o. administration of 2800 mg/kg to each of 4 rats elicited a mean 35 and 65% recovery at 4 and 8 hrs from the urine (plus carcass) and feces (plus GI content), resp. Approximately 70 - 90% is excreted in 24 hrs, of which 57 - 74% is renal and 17 - 36% intestinal. Concentrations of the label in cardiac blood were highly irregular, but tended to remain elevated for up to 4 hrs after 700, 1400 and 2800 mg/kg p.o. doses, with gradual decline toward 8 hrs post-treatment. The unabsorbed portion of sodium sulfate in the gastrointestinal tract—that which had already been absorbed and re-excreted—was ca. 57% (range 38 - 89%). A positive cathartic response, which began at 1400 mg/40 ml H_2O/kg p.o. was attributed to osmotic activity of unabsorbed sulfate: fluid retention in stomach, small gut, colon and cecum was elevated above control values. Intraluminal and i.v. studies in fistula dogs, mice and isolated ileal-colon segments from rat, rabbit and monkey confirmed the nonspecific bulk retention effect of sulfate. The increased gastrointestinal motility, which was detected even at sublaxative doses, was attributed to the increased bulk. No gross evidence of gastrointestinal mucosal inflammation was found.

12. Ref. 408 (sodium sulfate)

Isotonic sodium sulfate (25 to 50 ml) administered to two fasted dogs with both gastric and intestinal fistulas had no effect on gastro-intestinal activity (as measured by gastric and intestinal movement) when the salt was placed in contact with gastric mucosa. A five-fold hypertonic solution of Na_2SO_4 in contact with stomach mucosa increased intestinal activity by 30%. Placing the isotonic solutions of Na_2SO_4 into a loop of the intestinal fistula stimulated activity two-fold and increased the intraluminal fluid content. Twice isotonic solutions increased motility 8 times that of isotonic sodium chloride control levels.

13. Ref. 410 (ammonium sulfate)

The i.v. administration of isotonic solutions of ammonium sulfate in doses of 1 to 3 ml in 1 to 9 seconds elicits an EEG pattern similar to that elicited by acetylcholine and considered to be related to an acute anoxia. The EEG pattern after the slow injection of 1 ml of the ammonium sulfate consisted of an accentuation of the alpha rhythm followed by a short phase of decreased general activity, theta waves of a 2 second duration and a subsequent return to normal. Similar EEG patterns were obtained with 3 ml ammonium sulfate except that the period of electrical inactivity was more significant.

14. Ref. 440 (sodium sulfate)

Dialyzed human hemoglobin with a known level of oxygen saturation was subjected to increasing levels of sodium sulfate. Increasing the sulfate concentrations from 0.02 to 0.10 molar at a relatively constant hemoglobin concentration, oxygen pressure and pH (7.03 - 7.08) inhibited the affinity of hemoglobin for oxygen, reducing the fraction of oxygen-saturated hemoglobin from 81 to 31%.

It was suggested that the inhibiting effect may be related to a combination of the sulphate with hemoglobin rather than to the ionic strength of the solution although this latter correlation could not be excluded.

15. Ref. 463 (sodium sulfate)

Normal human volunteers maintained on a daily diet containing 10 to 20 meq of sodium chloride were infused for 8 hours daily with an isotonic solution containing an additional 155 meq of sodium. The anionic portion of the salt was alternated every seventh day between chloride ion and sulfate ion. The pH, CO_2 and electrolyte content of venous blood were determined and urine samples were collected daily.

Although individual urinary pH values were 0.05 to 0.48 units lower during sulfate infusion than during chloride infusion, the pooled values compared by a paired t-test did not reveal any significant differences. Qualitatively similar variations of titratable acid and net acid excretion due to sulfate administration were obtained. Significance was found when these data were compared to chloride periods rather than to control periods, the titratable acid being increased 1 - 12 meq/day (ca. 8%) and net acid excretion increasing 4 - 31 meq/day (ca. 8%) as compared to chloride values.

No variation in urinary ammonium or electrolyte excretion or blood composition due to sulfate infusion was noted. Continuously infusing sodium chloride for 3 days followed by a similar continuous infusion of sodium sulfate resulted in a slight increase in net acid excretion only. The authors conclude that the nature of the anion load does not play an important role in renal acid-base regulation. It was suggested that an interaction of independent mechanisms determine bicarbonate threshold, the excretion of fixed anions and cations and the synthesis of ammonia.

16. Ref. 475 (sodium sulfate)

Sodium sulfate (1.3 mg/ml) was found to activate a carboxylase-cocarboxylase system containing 30 μg/ml of synthetically prepared cocarboxylase.

17. Ref. 481 (ammonium and sodium sulfates)

Rat liver mitochondria incubated for 15 min. in a standard incubation medium containing sucrose, Tris-buffer, EDTA, $MgCl_2$ and orthophosphate, to which 2.5 to 10 mmolar ammonium sulfate has been added, loses endogenous potassium at an increased rate.

A 5 mmolar ATP exogenous energy source inhibited the potassium loss induced by 10 mM ammonium sulfate, while the respiratory sub-

strates succinate and glutamate prevented the loss of potassium induced by the ammonium sulfate.

Ammonium sulfate had no influence upon potassium uptake by previously depleted mitochondria regardless of the presence of ATP. Preincubation of mitochondria with ammonium sulfate did, however, abolish potassium uptake.

In contrast, sodium sulfate in the same concentrations as ammonium sulfate inhibited the loss of potassium in the absence of orthophosphate. With orthophosphate, potassium loss from the motochondria treated with sodium sulfate was no different than that observed in mitochondria treated with ammonium sulfate.

Ammonium and sodium sulfate prevented potassium loss from mitochondria in the absence of magnesium ion with or without orthophosphate.

The authors suggest that the increased potassium loss was due to increase in mitochondrial membrane permeability induced by sulfate ions. The increase in membrane permeability resulted presumably in a loss of endogenous substrates and nucleotides which reduced the ability of the mitochondria to maintain a sufficiently high energy level to prevent an acceleration of the potassium loss.

18. Ref. 506 (ammonium sulfate)

The activity of DNA-dependent rat liver nuclei RNA polymerase, as measured by the incorporation of labeled ATP into RNA, incubated in the presence of 5% ammonium sulfate was increased two-fold over a 25 min. period as compared with polymerase activity in the absence of ammonium sulfate. Polymerase activity varied directly with sulfate concentration.

A 10% concentration of ammonium sulfate (0.4M) in the incubation mixture was found to result in optimal increases of RNA polymerase activity when stimulated by Mn or Mg, with Mn the preferential potentiator. Inclusion of ammonium sulfate in the incubation mixture was found to influence the composition of the RNA synthesized, resulting in DNA-like RNA, an RNA of relatively higher molecular weight than that obtained in the absence of ammonium sulfate. Nuclei incubated in the absence of ammonium sulfate resulted in ribosomal-like RNA.

19. Ref. 508 (sodium and ammonium sulfates)

A very low order of activation of beef liver glycerate dehydrogenase was obtained in enzyme solutions incubated with sodium or ammonium sulfate (0.028M).

20. Ref. 509 (sodium and ammonium sulfates)

Bovine pancreatic ribonuclease A activity with c-cytidilic acid as substrate fell 50% with the addition of 0.5 molar $(NH_4)_2SO_4$ to the incubation mixture. Activity began increasing again above 1 molar salt and reached its original activity at 3 molar $(NH_4)_2SO_4$. RNAase A dimer curves reproduced those for the monomer. The reconstituted RNAase (S') curve also simulated that for RNAase A up to 2 molar, after which the slope rose more sharply, and in 3 molar $(NH_4)_2SO_4$ the activity was 60% greater than without this salt. S-protein-S-peptide (constituents of RNAase S) activity was also 50% inhibited at 0.5 - 1 molar concentrations, but activity fell to 20% of the original at 3 molar $(NH_4)_2SO_4$, suggesting conformational alteration. RNAase activity declined linearly with increasing sulfate concentration to extinction at 2 molar during incubation with yeast RNA.

The Na_2SO_4 slopes paralleled those for the NH_4 salt, confirming that divalent anions were the strongest inhibitors at low, and the strongest activators at high, concentrations. Moreover, there was some evidence that polymeric enzyme aggregates would be dissociated at high salt concentrations. It was concluded that divalent sulfates interfere with the stability of tertiary or quaternary structure and that the active RNAase form in concentrated salt solutions differs from the "native" form in dilute solutions.

21. Ref. 515 (sodium sulfate)

Ten toads prepared for collecting subcutaneous lymph received 10 ml/kg body weight of a 5% sodium sulphate solution i.v. Whereas the lymph flow is increased in the presence of sodium sulphate (7.73 ml/2 hours) as compared to control values (5.58 ml/2 hours), the amount of crystal violet dye found in the lymph as a measure of capillary permeability, was remarkably decreased by a factor of 10.

22. Ref. S4 (sulfate)

In human erythrocytes incubated in plasma with 0.5% glucose or hemolysates with 0.5% glucose the concentration of 2,3-diphosphoglycerate is higher over an 8 - hour period, when 8m molar sulfate is added to the system. The same holds true in glucose free hemolysates or suspensions, suggesting that sulfate inhibits diphosphoglycerate phosphatase. The glucose utilization rate also increased by ca. 1.7x and lactate formation by ca. 1.5x in the presence of 5m molar sulfate, in the plasma - erythrocyte - 0.5% glucose system.

23. Ref. S5 (sodium sulfate)

The addition of sodium sulfate in various concentrations to mouse L-strain fibroblasts (NCTC 929), mouse parenchymal liver cells (NCTC 1769) and HeLa cultures for various periods of time caused little inhibition of fibroblast growth during the first 7 days at 200 or 100 mg%, moderate inhibition (14 - 25%) of mouse liver cell growth through the first 7 days at 200 mg% while at 100, 50 and 10 mg% an increase in cell growth occurred. HeLa cells ceased to grow significantly after 5 days at 50, 100 and 200 mg %.

24. Ref. S6 (sodium sulfate)

ATP sulfurylase isolated from baker's yeast liberated small amounts of adenosine phosphosulfate (APS) and phosphate from ATP in the presence of sodium sulfate substrate. Addition of APS-kinase increased the liberation of phosphate ten-fold. In the presence of sodium sulfate the sulfurylase enzyme forms an AMP-sulfate end product.

IV. Drug interactions

1. Ref. 326 (sodium sulfate)

Male White-Plymouth Rock X Vantress crossbred chicks five weeks old were fed a basal diet containing 0.7% chlortetracycline (CTC). Increasing levels of calcium in the diet from 0.17 to 1.4% resulted in a decrease in CTC absorption which was reversed by the addition of various levels of sodium sulfate, 2% being most effective. It was suggested that sulfate ion interacts with intestinal calcium content, thus preventing formation of an antibiotic-calcium complex which inhibits CTC absorption.

2. Ref. 131 (sodium sulfate)

Both fasted and fed female Golden Labradors between 17 and 24 months of age were infused with 3 parts of 5% creatinine and 2 parts of sodium sulfate (50 mmolar) at 0.75 and 1.0 ml/min. Increases of 30 to 50% in glomerular filtration rate above initial values were observed in the fasted dog. Sodium sulfate administration yielded an increase in phosphate filtered load as measured from heparinized plasma. Infusion of 5% creatinine alone did not result in a substantial increase in phosphate excretion. Similar levels of phosphate filtered loads were also obtained in fed state.

3. Ref. 246 (sodium sulfate)

Sodium sulfate (1%) added to milk was proven to be effective in the treatment of vitamin D intoxication and idiopathic hypercalcemia. There was a 50% reduction in calcium retention in rats after treatment, due to inhibition of calcium ionization and subsequent suppression of calcium absorption from the gut.

4. Ref. 367 (potassium sulfate)

The anesthetic effect of a 0.05% cocaine solution s.c. into the foot pad of frogs lasting 5 - 20 min. was extended to 100 - 240 min whtn 0.4% K_2SO_4 was added to the solution. No potentiation was observed in the conjunctiva or nasal mucous membrane.

5. Ref. S1 (sulfuric acid)

Exposure of guinea pigs for 8 hours to a combination of 8 mg/cu.m of sulfuric acid mist and 89 ppm sulfur dioxide resulted in a decrease of body weight from approximately 240 to 215 gms the day following exposure. Exposure 4 days later to the same concentrations for an

additional 8 hours inhibited growth entirely. Exposure to 8 mg/cu m of sulfuric acid mist alone for 72 hours resulted in a weight loss during exposure which was quickly regained after exposure.

Lung pathology showed some areas of mainly hilar consolidation but no hepatization. Nor were noticeable respiratory effects observed after exposure to the sulfuric acid mist alone.

V. Consumer Exposure Information

Summary of possible daily intake of all sulfate additives from NAS/NRC comprehensive GRAS survey, Oct., 1972, tables 2 and 13A.

Possible daily intake in 2 - 65 yr age group, as mg SO_4

Salt	Food Category	Average*	High A**	High B***
Ammonium Sulfate				
	Baked goods	33.2	49.4	70.6
	Gelatins, pudding	11.2	28.8	14.9
Calcium Sulfate				
	Baked goods	113.2	168.1	264.3
	Milk products	0.4	1.4	0.5
	Frozen dairy	5.7	13.8	10.4
	Processed vegetables	211.2	355.8	211.2
	Confectionaries, Frostings	3.0	8.0	6.4
	Sweet sauce	1.1	2.9	1.4
	Gelatins, puddings	59.3	152.6	59.4
	Alcoholic beverages	7.1	20.7	10.3
Potassium Sulfate				
	Nonalcoholic beverages	6.1	16.2	9.1
Sulfuric Acid				
	Nonalcoholic beverages	4.3	12.2	4.3
Total		455.8	829.9	662.8

Possible daily intake in 0 - 5 mos. age group, as mg SO_4

Salt	Food Category	Average*	High A**	High B***
Calcium Sulfate				
	Baked goods	2.8	3.7	6.5
	Processed vegetables	3.5	10.4	3.5
	Gelatins, puddings	5.8	7.8	5.8
Total		12.1	21.9	15.8

Possible daily intake in 6 - 11 mos. age group, as mg SO_4

Salt	Food Category	Average*	High A**	High B***
Ammonium Sulfate				
	Baked goods	6.1	12.6	13.1
	Gelatins, puddings	7.0	21.2	9.3

Calcium Sulfate				
	Baked goods	20.5	42.7	48.9
	Milk products	0.7	3.3	0.8
	Processed vegetables	59.6	139.2	59.6
	Gelatins, puddings	37.2	112.8	37.2
Totals		131.1	331.8	168.9

* Usual additive use and mean consumption
** Usual additive use and high consumption
*** Maximum additive use and mean consumption

A man could intake 1.28 gm SO_4 daily if the maximum employed sulfate additive was used in foods in the following extreme diet.

Food category	consumption	SO_4 (mg)
Confectionaries (3.0% $CaSO_4$)	500 gm	1065
Processed vegetables (0.35% $CaSO_4$)	500 gm	124
Baked goods (0.27% $CaSO_4$)	500 gm	89
Total		1,278

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APPENDIX B

(full text copy of all papers used in the summary of the monograph)

to

SULFATES

A Monograph

Prepared for
Department of Health, Education and Welfare
Public Health Service
Food and Drug Administration
Rockville, Maryland

by

Dr. William Goldman

FIRL Project No. 80G-C3378-01

February 5, 1973

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Drury, Horsfall et Munly (3*) ont noté aussi la fréquence avec laquelle on peut déterminer la fibrillation ventriculaire au cours de l'intoxication quinidinique, et ils supposent que le fait se produit au moment où l'empoisonnement n'est pas encore très profond et où, par conséquent, l'excitabilité du muscle est encore assez forte pour qu'il puisse réagir. Nous avons, par contre, observé la fibrillation spontanée après des doses très intenses des divers alcaloïdes ci-dessus considérés et l'hypothèse des auteurs anglais ne paraît pas trouver ici son application. A la question, donc, de savoir pourquoi l'action empêchante de ces alcaloïdes sur la fibrillation du muscle auriculaire, ne s'exerce pas sur les ventricules, nous sommes pour l'instant dans l'impossibilité de répondre.

La concentration des solutions dans l'intestin. Les seuils d'absorption,

par Ch. Achard et A. Leblanc.

L'un de nous a étudié autrefois, avec L. Gaillard et A. Ribot, les échanges qui s'accomplissent dans le péritoine et dans l'intestin quand on introduit dans ces cavités des solutions de divers corps, à différents degrés de concentration. Au cours de ces recherches, un fait, que l'un de nous a signalé brièvement dans une autre publication (1), avait été observé : c'est que, dans l'intestin, l'introduction de certains corps en solution hypotonique peut être suivie d'une concentration de ces substances, de sorte que l'intestin semble pouvoir concentrer pour l'absorption, comme le rein concentre pour l'excrétion.

C'est ce fait que nous avons entrepris d'étudier dans de nouvelles expériences. Celle-ci ont été faites chez le Chien ; les précédentes avaient porté sur le Lapin. Les animaux étaient à jeûn depuis 48 heures et anesthésiés peu profondément par le chloralose ou le mélange de chloral et morphine. Les anses intestinales étaient choisies dans la dernière partie de l'intestin grêle. Quand plusieurs anses voisines étaient utilisées chez le même animal, elles étaient toujours isolées les unes des autres par une double ligature.

Lorsqu'on introduit dans ces anses intestinales des solutions hypertoniques, on constate que, à mesure que se fait l'absorption, la concentration baisse. Mais si l'on introduit des solutions hypotoniques, des différences apparaissent. Il est des corps qui ne subissent pas de concentration : l'urée, l'alcool ; il en est d'autres qui se concentrent : le NaCl, le sulfate de soude, le glycose, le glycocolle, l'alanine. C'est ce qu'on peut voir sur le tableau suivant qui présente les exemples les plus significatifs tirés de nos recherches :

(3*) Drury-Horsfall et Munly. *Heart*. t. IX, n° 4, 1922, p. 365.

(1) Ch. Achard. Le partage du liquide entre les milieux vitaux. *Semaine médicale*, 10 juillet 1907, p. 325.

	Solution introduite				Liquide retrouvé	
	Taux de la substance p. 1.000		Volume, en c.c.	Temps écoulé en minutes	Taux de la substance, p. 1.000	Volume, en c.c.
Urée	10,5	Δ = — 0°30	40	30	3,55	15
Alcool éthylique	0,077	Δ = — 0°29	50	30	0,042	30
NaCl	4,8	Δ = — 0°32	40	20	7,25	25
Sulfate de soude	7,6	Δ = — 0°29	40	30	15,75	16
Glycose	33,3	Δ = — 0°24	40	35	41,66	26
Glycocolle	10,8	Δ = — 0°29	50	30	13,9	22
Alanine	10,8	Δ = — 0°25	50	30	21,1	30

Remarquons que l'urée et l'alcool éthylique sont des corps qui s'éliminent par le rein obligatoirement en quelque sorte, à quelque taux qu'ils se trouvent dans le sang, tandis que les autres corps ci-dessus s'éliminent seulement au-delà d'un certain taux sanguin. On peut dire, par conséquent, comme on le dit pour l'excrétion rénale, que l'urée, l'alcool éthylique n'ont pas de seuil d'absorption intestinale, tandis que les autres corps en ont un.

En réalité, alors que la concentration atteint dans l'intestin un taux souvent assez bas, le passage de la substance dissoute peut commencer, mais avec une très faible intensité, de sorte que l'eau s'absorbant plus vite, la substance se concentre et l'absorption s'accroît ; puis, à un certain degré de concentration, l'absorption se fait d'une manière en quelque sorte obligatoire ; si bien qu'il y a peut-être deux seuils : l'un, plus bas, d'absorption débutante, et l'autre, plus haut, d'absorption obligatoire.

Le seuil d'absorption intestinale n'est pas le même pour les diverses substances. Il paraît varier suivant les animaux et, peut-être même, dans le cours d'une même expérience et subir encore l'influence des modifications de la membrane. On ne saurait en être surpris, car la paroi de l'intestin est une membrane complexe, pourvue non seulement, comme les tubes du rein, d'un épithélium actif, mais d'organes glandulaires dont la sécrétion peut intervenir pour modifier le contenu de la cavité muqueuse.

On ne saurait donc déterminer ces seuils avec une grande précision en mesurant le taux de concentration que peuvent atteindre au bout d'un certain temps les substances introduites en

solutions hypotoniques. Provisoirement, pour fixer les idées, nous estimons que le seuil de l'absorption obligatoire est, d'une façon tout à fait approximative, pour le NaCl, à 7,6 p. 1.000 ; pour le sulfate de soude, à 16 ; pour le glycose, à 40 ; pour le glycocolle, à 14 ; pour l'alanine, à 33.

La question des seuils de passage à travers les membranes, surtout étudiée pour l'excrétion rénale, se pose aussi pour l'absorption, et il peut être intéressant de l'envisager en quelque sorte par une autre face que celle de l'excrétion. Nous connaissons mal en quoi consistent ces seuils, car dire qu'il y a ou non, pour une substance donnée, un seuil de passage, c'est constater un fait, mais non l'expliquer. Il peut n'être pas inutile d'aborder cette étude dans d'autres conditions que celles de l'élimination rénale.

Caractérisation microchimique d'un composé sulfuré dans la cellule épithéliale granuleuse du poumon,

par E. Fauré-Fremiet et J. Dragoiu.

Nous avons montré, en 1920 (1°), que la fixation prolongée du tissu pulmonaire avec un mélange de formol et de chlorure de cadmium (2°) donnait une coloration variant du jaune clair au brun intensément localisée à la surface des granulations lipoïdes de la cellule épithéliale globuleuse du poumon.

Nous avons vérifié dans le poumon de Mouton fœtal ou adulte et dans celui du Cobaye ou du Rat, que cette réaction est très caractéristique de la cellule épithéliale granuleuse bien connue aujourd'hui (3°).

La spécificité de cette réaction est particulièrement intéressante lorsque l'on étudie l'histogénèse du poumon fœtal (4°). Cazzaniga (5°) appliquant notre technique à l'étude du poumon humain fœtal a pu vérifier nos résultats et obtenir la réaction

(1°) Fauré-Fremiet, Dragoiu et du Vivier de Streel. Sur une réaction microchimique de la cellule épithéliale pulmonaire. *Bull. de la Soc. de chimie biologique*, t. II, p. 166.

(2°) Formol à 10 p. 100 : 100; $CdCl^2$: 3. Le Dr Bétancès nous signale que ce fixateur conserve remarquablement les granulations des leucocytes.

(3°) Gilbert et Jomier, Granel, Guieysse-Pellissier, Fauré-Fremiet, etc...

(4°) Dans le poumon adulte la teinte obtenue peut prêter à confusion avec celle des grains de pigment ocre que l'on peut rencontrer dans les cellules à poussière.

(5°) Cazzaniga. Sulle moderne direttive di accertamento integrale dei valori di vitalità. Lo sperimentale, t. LXXVI, 1922.

« cadmiophile » de la cellule granuleuse, lorsque celle-ci apparaît à la fin de la vie fœtale.

En ce qui concerne la signification de cette réaction, l'étude des coupes obtenues après congélation montre que la coloration jaune ou brune est due à un précipité inorganique qui se forme progressivement au niveau des granulations lipoïdes de la cellule granuleuse. Ce précipité est soluble dans l'acide nitrique, et ses différents caractères nous ont fait penser qu'il est constitué par du sulfure de cadmium.

Nous avons vérifié, d'autre part, au cours de notre première étude, que l'extrait éthéré des lipoïdes pulmonaires entraine du soufre, sous une forme que nous n'avons pas caractérisée, et que, de toute manière, la formation du sulfure de cadmium ne se produit que lentement et *en présence de formol seulement*.

Nous avons tenté d'obtenir cette réaction avec un métal donnant un sulfure noir ; la présence nécessaire de formol limite les possibilités et nous avons essayé l'acétate de plomb.

Le tissu pulmonaire a été fixé par injection dans la trachée du mélange suivant :

Formol à 10 p. 100	100
Acétate de plomb	5

Ce liquide est acidifié légèrement ($P_H = 3,3$ à $3,5$) par l'acide acétique.

Après 15 à 20 jours de séjour dans ce liquide (6°) les granulations lipoïdes de la cellule granuleuse sont intensément *colorées en noir*, ou plus exactement recouvertes d'une pellicule noire. La couleur du précipité est donc bien celle du sulfure du métal employé.

La réaction à l'acétate de plomb semble plus longue à obtenir que celle au chlorure de cadmium ; mais, au point de vue cytologique, elle est peut-être préférable, surtout si l'on veut effectuer des coupes à la paraffine.

Dans le cas de la fixation au formol-chlorure de cadmium, la disparition de la masse lipoïde des granulations au cours des passages dans l'alcool et le xylol provoque fréquemment la désagrégation du précipité de sulfure superficiel ; d'autre part, celui-ci est parfois d'un jaune transparent qui tranche mal en présence de divers colorants histologiques ; au contraire, le sulfure de plomb reste parfaitement opaque et dessine nettement en noir la forme des globules lipoïdes après leur dissolution par les réactifs.

(6°) Ce mélange reste parfaitement stable aussi bien que celui de formol et de chlorure de cadmium.

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other characters. It may be significant that the change from *Uria aalge albionis* to *Uria a. aalge* corresponds with the first zone of change in percentage of breeding, but it does not seem safe to postulate a causal connexion between the ebb in polymorph frequencies and that in melanin pigmentation on the present evidence.

The genetic situation involved is of some interest. From the non-existence of intermediate forms it may be assumed that the control is unifactorial. Whether it behaves as dominant or recessive is, however, less clear, and only breeding experiments or ringing of wild populations could decide this.

This method of describing sub-species and forms makes a useful addition to the usual taxonomic procedure and gives important information as to the processes of species differentiation, the methods by which isolation arises in a widespread group and the speed with which genes may diffuse across such barriers.

H. N. Southern.

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University of Oxford.
Oct. 27.

[1] Huxley, J. S., Nature, 142, 219 (1938).
[2] Johnson, R. A., *Aut*, 35, 56–61 (1938).

Tellurium Tetrafluoride

The formation of a white solid which accompanies the main product tellurium hexafluoride when fluorine is passed over tellurium has often been noticed but its composition has not been settled (Moissan[1], Prideaux[2]). Yost and Claussen[3] showed that the same or a similar substance is formed by heating the hexafluoride with tellurium in sealed glass tubes, but they were unable to identify the product, which they state is probably the difluoride, TeF_2.

We have re-investigated the reaction using sealed tubes of both glass and silica. With the hexafluoride alone in the tube, no reaction occurs and the density of the gas remains unchanged. With tellurium present, a white solid is formed at a temperature of about 200° C. The ratio of fluorine to tellurium in this solid is not constant but decreases with the time the tube is heated, until after heating for several days, the fluorine content becomes very small and the solid approximates in composition to TeO_2. At the same time the gas pressure in the tube rises and finally attains a value 1·5 times the initial pressure. The gas, originally pure TeF_6, is converted completely into SiF_4, as shown by vapour density measurements. The most probable explanation of these facts seems to be that the first product of the reaction is the formation of a new compound, tellurium tetrafluoride, which then reacts with the silica giving silicon fluoride and tellurous oxide:

$$(1)\quad 2TeF_6 + Te = 3TeF_4.$$
$$(2)\quad 3TeF_4 + 3SiO_2 = 3TeO_2 + 3SiF_4.$$

Further investigation has confirmed the correctness of this interpretation. We have found that the product of the first reaction can be isolated by using a tube composed of pure crystalline alumina. This material, as supplied by the Thermal Syndicate, Ltd., is non-porous and is the only one we have found which is not attacked by this reaction.

In these conditions, the tellurium hexafluoride is absorbed completely by the tellurium at about 200° C., forming a colourless solid which crystallizes in fine needles on the walls of the tube. On exposure to ordinary air, the solid hydrolyses very rapidly with evolution of hydrogen fluoride. By dissolving this product rapidly in aqueous potash, we have been able to determine the ratio F : Te in the substance. Two independent experiments showed it to approximate closely to 4 : 1.

Quite recently we have been able to prepare larger amounts of the white crystalline material free from excess of tellurium, and to analyse it completely. As a mean of two experiments, we find for its composition: Te = 62·5 per cent, F = 38·7 per cent. The formula TeF_4 contains Te = 62·6 per cent, F = 37·4 per cent. The fluorine was estimated as lead chlorofluoride after precipitation of tellurium as dioxide—a method which we have found to give rather high results when used for the estimation of this element in compounds of tellurium and fluorine.

The results can therefore be regarded as satisfactory, and there can be little doubt that the white solid we have obtained is a new fluoride of tellurium, TeF_4, which on account of its reactive behaviour with glass and silica and its instability in presence of traces of moisture has not been isolated previously.

Further experiments on the properties and behaviour of this substance are in progress.

G. A. R. Hartley.
T. H. Henry.
R. Whytlaw-Gray.

Chemistry Department,
University,
Leeds.
Nov. 1.

[1] Moissan, *Ann. Chim. Phys.*, 24 (6), 239 (1891).
[2] Prideaux, *J. Chem. Soc.*, 89, 320 (1906).
[3] Yost and Claussen, *J. Amer. Chem. Soc.*, 55, 885 (1933).

A.H.W. Aten

Fate of the Sulphate Radical in the Animal Body

Phosphorus enters as phosphate in the numerous compounds in which it is to be found in the animal body; in connexion with the investigations carried out in recent years concerning the fate of ingested phosphorus atoms in the organism, it seemed to be of interest to determine whether or not, in the course of the numerous metabolic processes in which phosphorus is involved, the phosphate radical exchanges its *oxygen* content with other oxygen atoms present in the body. This question could be answered by injecting into an animal sodium phosphate which contained heavy oxygen (^{18}O) as an indicator and then determining if the phosphate recovered in the urine, for example, contained more than the normal amount of ^{18}O.

As, however, it was recently found[1] that 'heavy-oxygen phosphate' can be obtained by dissolving sodium phosphate in 'heavy-oxygen water' and vice versa, it is apparent that the oxygen atoms present in phosphate radicals exchange their places freely in water and there can be scarcely any doubt that the probability is extremely small of a phosphate radical leaving the body coupled to the same oxygen atoms with which it entered. Sulphate ions, on the other hand, have been found[2] to exchange oxygen atoms either not at all or at a very slow rate with neutral water, even at 100° C., and it seemed of interest, therefore, to investigate whether sulphate ions during their circulation in the body participate in chemical reactions which loosen the oxygen bonds sufficiently to make an oxygen exchange possible.

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In the experiments we wish to report here, sodium sulphate containing heavy oxygen was prepared from heavy-oxygen water, kindly presented to us by Prof. Urey[4], having a density 740 parts in a million greater than that of normal water. The reaction used for the preparation of the 'heavy sulphate' was that which takes place between SO_2Cl_2 and heavy-oxygen water in the presence of traces of iodine as a catalyst. 1 gm. of the dry material, converted into 50 c.c. of solution, was injected into a rabbit; we are very grateful to Miss Lindberg, of Prof. Krogh's laboratory, for making the injections. The urine of the rabbit was then collected for 24 hours, its sulphate content recovered as barium sulphate, the oxygen content of the latter converted into water, and the density of this determined. The preparation of water from the oxygen of the sulphate was carried out in the following way. The barium sulphate precipitate was dried at 400° C. in a stream of nitrogen and then reduced with purified carbon at 900° C.; the gases evolved were mixed with a great excess of hydrogen and stored over oil in a gasometer; and, finally, the gas mixture was led over a nickel catalyst at 310° C. and the water formed collected. The density determination was kindly carried out by Mr. O. Jacobsen, using Linderstrom-Lang's floating-drop method.

Should the sulphate oxygen, during its stay in the animal, enter into exchange reactions with other oxygen atoms present in very great excess in the body, the oxygen of the heavy radicals would be replaced by normal oxygen atoms and the water prepared from the sulphate recovered from the urine would show the density of normal water. If, on the other hand, the sulphate ions injected retain the oxygen atoms with which they start, the water prepared from the urine sulphate should show an excess density of 370 parts per million if no secretion of normal sulphate took place. The water prepared from the sulphate isolated from the urine after injecting heavy-oxygen sulphate has shown a very appreciable density excess—240 parts per million. When comparing this value with the one calculated on the assumption that no exchange of sulphate oxygen took place, we must consider the following fact. Besides the heavy-oxygen sulphate—0·84 gm. of sodium sulphate being secreted in all during the day following injection—the urine contains also sulphate, even when no injection is given, the amount of which we found to correspond to 0·23 gm. per day. The latter is normal sulphate and its presence reduces the density excess of the water prepared from the urine sulphate.

From the high density found for the water prepared from urine sulphate, one must conclude that most of the individual sulphate ions injected into the rabbit are recovered in their original form, and from this it follows that at least the greatest part of the sulphate administered leaves the body unchanged, and also that none or only a small part of the ingested ions exchange as such with other sulphate ions present beforehand in the tissues.

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G. Hevesy.

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Copenhagen.

[1] Blumenthal and Herbert, *Trans. Faraday Soc.*, 33, 849 (1937).
[2] Datta, S. C., Day, J. N. E., and Ingold, C. K., *J. Chem. Soc.*, 1968 (1937).
[3] Hoffmann and Urey, *Ind. Eng. Chem.*, 29, 531 (1937).
[4] Manian, Urey, and Bleakney, *J. Amer. Chem. Soc.*, 56, 2601 (1934).

Distribution of Phosphorus in the Leg Bones of Chickens

In rickets, as is generally known, the total phosphorus content of the bones is diminished. We have started now to investigate the distribution of phosphorus in the leg bones of normal and rachitic chickens with a radioactive phosphorus isotope as an indicator.

The active phosphorus used was prepared by Dr. F. A. Heyn, of the Philips Lamp Works at Eindhoven, with a Philips neutron generator[1]. A fixed quantity of the active phosphorus was injected intraperitoneally as an aqueous solution of sodium phosphate of *p*H 7·2. The chickens were decapitated 22 hours after the injection and quickly sectioned. The leg bones were then dissected and cleaned. One of the leg bones of each bird then was divided into three parts, namely, the proximal epiphysis, the distal epiphysis and the diaphysis. The two epiphysial parts of the bone were then carbonized together in an oven at 200° C.; the same was done with the diaphysis. Preparations for the determination of the radioactivity were made in the manner described previously[2]. In addition, a part of the residue of carbonization was used for the estimation of the total phosphorus according to Fiske-Subbarow.

As was to be expected, the provisional figures from eight chickens showed that the phosphorus content of the dried matter in the epiphysial part and in the diaphysial part is larger in normal than in rachitic chickens. Furthermore, it was observed that both in normal and in rachitic chickens, the phosphorus content from the diaphysial part of the bone seems to be larger than that from the epiphysial part of the same bone.

With regard to the distribution of the active phosphorus administered, it was observed that, both in the normal and in the rachitic chickens decapitated 22 hours after the injection of the labelled phosphorus, the quantity of the active phosphorus in 1 mgm. of bone phosphorus was larger in the epiphysis than in the diaphysis. Furthermore, it was observed that both the epiphysis and the diaphysis from the rachitic birds, decapitated 22 hours after the injection of the labelled phosphorus, contained a much larger quantity of the active phosphorus in 1 mgm. bone phosphorus than the normal chickens.

The second leg bone of each chicken was not carbonized, but after being cleaned was placed on a double coated X-ray film. It remained on this film for some days, according to the quantity of radioactive phosphorus injected. The film was then

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	Glycine, Alanine	Leucine or Isoleucine
	Glycine, Alanine, Leucine or Isoleucine	Aspartic acid, Valine
e, . e or cine	Aspartic acid	

eriment.

d its hydantoin at pH 8. xtra degradation products ne extra cleavages involved no groups of hydroxyamino products could not be d.

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Utilization of S^{35}-Labelled Sodium Sulphate in the Synthesis of Chondroitin Sulphuric Acid, Taurine, Methionine and Cystine

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The high incorporation of S^{35}-labelled sodium sulphate in sulpho-mucopolysaccharides of mammalian tissues has been demonstrated by different workers [1-4]. In several autoradiographic studies [5-8] on the sulphate fixation in different mammalian organs, it was shown that the main uptake of sulphate took place in those tissues, such as cartilage, the aorta, sclera, cornea and intestinal mucosa, which are known to contain large amounts of sulpho-mucopolysaccharides. On the other hand, it is known that S^{35} fed to rats as sulphate does not appear in cystine [9]. These facts thus speak in favour of the opinion that S^{35} administered as sulphate is mainly incorporated as ester sulphate in mucopolysaccharides.

In the present investigation a comparative study was made of the utilization of sodium sulphate in the synthesis of chondroitin sulphuric acid, taurine, methionine and cystine.

Four groups of adult white rats *, each group comprising 20 rats weighing 250–300 g, were given intraperitoneal injections of 1.5 mg of S^{35}-labelled Na_2SO_4 ** in 0.5 ml of distilled water (3.5×10^6 counts per minute per cm^2 measured as $BaSO_4$ at infinite thickness). The different groups of animals were sacrificed 2, 4, 16, and 24 hours, respectively, after the injection. The livers of all the animals in a single group were pooled and likewise the ribs with the intercostal muscles. The material thus obtained was handled in the following way.

* The same animals as those used for previous experiments [5].

** Obtained from *A.E.R.E.*, Harwell, England.

Chondroitin sulphuric acid was prepared from the cartilage of the ribs by applying the method of Jorpes [10] as modified by Strandberg [11]. The details of the method used and the analytical figures of the preparations obtained are given in a previous paper [5].

Isolation of taurine. The livers from each group of animals were minced into alcohol and the pooled livers ground in a Turmix blender (Swiss), filtered, and dried with more alcohol. The material was then extracted for two hours with boiling 3 : 1 alcohol : ether. The alcohol and alcohol-ether extracts were pooled and evaporated to dryness at reduced pressure. The residue was dissolved in a minimum of chloroform; this was poured into a large volume of acetone, and the precipitate filtered off. The material was twice more dissolved in chloroform and precipitated with acetone. The precipitate was then hydrolyzed in boiling 6 *N* HCl for 24 hours. After cooling, the solidified fatty acids were filtered off. The filtrate was evaporated to dryness, and the excess hydrochloric acid removed by repeated evaporations *in vacuo*. The final residue was dissolved in *N* HCl and applied to a column of the cation exchange resin Dowex 50 in the H^+ form. The length of the column was 20 cm and the diameter 4 cm. The separation was carried out with *N* HCl as the eluting solvent. The taurine emerges as a sharp peak very early in the chromatogram. This peak occurs between the solvent front and the peak of the first amino acid (aspartic acid). The fractions containing taurine were pooled and evaporated to dryness. Excess acid was removed by repeated evaporations *in vacuo*. The taurine was then chromatographed on starch, using the solvent system 2 : 1 propanol : 0.5 *N* HCl to check the identity and purity [12]. The taurine was then put through a small Dowex 50 column (20 cm × 0.9 cm) from which it was eluted in *N* HCl in a highly pure form. The identity and purity were further verified by paper chromatography.

Isolation of methionine and cystine. The liver material remaining after extracting the lipid components was extracted twice with 5% trichloroacetic acid (TCA) at 90° C for 15 minutes according to Schneider [13]. The residue was dried with alcohol and ether. About 5 g of this TCA-protein was hydrolyzed by boiling for 24 hours in a solution 6 *N* with respect to HCl and 50 per cent with respect to formic acid. The solution was freed from excess acid by repeated evaporations *in vacuo* and applied to Dowex 50 for the separation of the amino acids [14]. The column was 60 cm × 5 cm. To obtain a better resolution of methionine and

Sulf.

Substance	Radioactivity in counts/min./cm² at different times after injection			
	2 h	8 h	24 h	48 h
Chondroitin sulphuric acid isolated from the costal cartilage	1392	2136	2953	2933
Taurine isolated from the liver	32	68	53	35
Methionine isolated from the liver	—	—	3	—
Cystine isolated from the liver	—	—	4	—

isoleucine, the column was run at 70° C, which resulted in a complete separation of these two amino acids. The fractions corresponding to methionine and cystine, respectively, were pooled and evaporated to dryness. Both were then re-run on small Dowex 50 columns (20 cm × 0.9 cm) from which they were eluted in a few milliliters of 2.5 *N* HCl. No further purification of methionine and cystine was undertaken. As in the case of taurine, the identity and purity were checked by paper chromatography.

The *radioactivity* in the different samples was measured in the following manner. 80 mg of each of the preparations of chondroitin sulphuric acid were hydrolyzed with 6 *N* HCl for 4 hours on a boiling water bath and the sulphate precipitated as $BaSO_4$. In the case of taurine, methionine and cystine, $BaSO_4$ was precipitated after oxidation of about 40 mg of the substance with sodium peroxide according to Bailey. The different samples of $BaSO_4$ were then plated as described elsewhere[3] and measured by means of a Geiger-Müller counter with a 2.1 mg per cm² mica end-window tube. The radioactivity of the samples was expressed as counts/min./cm² at infinite thickness.

The results are given in the table. In agreement with earlier investigations [1,3] the incorporation of S^{35} in the sulphate group of chondroitin sulphuric acid of cartilage was very high after a single injection of labelled sulphate. A very low although significant uptake of S^{35} was demonstrated in taurine, isolated from the liver. The maximum uptake in chondroitin sulphuric acid and taurine was reached in 24 and 8 hours, respectively. As also shown by Tarver and Smith [9], S^{35} administered as sulphate was not utilized in the synthesis of cystine, nor did any incorporation of S^{35} take place in methionine.

The present investigation seems to afford further support to the opinion that the main uptake of administered sulphates occurs in mucopolysaccharides containing ester sulphates.

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different times after injection	
24 h	48 h
2953	2933
53	35
3	—
4	—

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Exchange of the Acetyl Group of Chondroitin Sulphuric Acid in Slices of Cartilage

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In an earlier paper[1], we have reported on the exchange of the sulphate group of chondroitin sulphuric acid in slices of cartilage. The object of the present study was to investigate the exchange of the acetyl group by means of the previously described slicing technique, after addition of sodium acetate labelled with C^{14} in the methyl group. A relatively low but definite uptake of C^{14} was demonstrated for both the methyl and the carboxyl groups of the acetic acid.

Fresh costal cartilage from 1 to 3-day-old calves was freed from muscles, connective tissue and perichondrium, and sliced to a thickness of approximately 0.2 mm. The slices were incubated in portions of 10 g with shaking in an oxygen-carbon dioxide atmosphere (93.5 % O_2 plus 6.5% CO_2) at 37° C in Erlenmeyer flasks, each containing 50 ml of Krebs' solution [3], sodium acetate labelled with C^{14} in the methyl group *, and 50 000 I.U. of penicillin. The slices were taken at different times and boiled for five minutes. Sodium salts of chondroitin sulphuric acid were then prepared from all the samples, according to a method described elsewhere [5]. The yield was 200 to 300 mg from each portion of 10 g. The figures obtained on analyzing the different preparations are given in the table.

150 mg of the preparations obtained were hydrolyzed under reflux with 10% by volume of sulphuric acid for three hours at 100° C. The acetic acid was then isolated by distillation. 0.05 *N* NaOH was added to pH 7 (glass electrode control), and the solution evaporated to dryness. The sodium acetate was then degraded according to the method of Phares [4]. After plating of the barium carbonate obtained, employing a filtering technique, the radioactivity of each of the carboxyl and methyl carbons was measured in a Geiger-Müller counter with a 2.1 mg per cm² mica end-window tube.

Three different experiments, A, B, and C, were carried out in which 1.6 mg, 0.5 mg, and 1.0 mg, respectively, of sodium acetate labelled with C^{14} in the methyl group and with an activity of 2.5×10^6 c.p.m. per cm² (measured as $BaCO_3$ at infinite thickness) were added. The rate of uptake of C^{14} in the methyl and carboxyl carbon of the acetyl group of the chondroitin sulphuric acid was determined. The periods of incubation varied from 0 to 30 hours. In two control experiments the incubation was performed with cartilage slices which had been boiled for five minutes prior to incubation with 1.0 mg of labelled acetate.

The results of the experiments are shown in the figure. Curves A, B, and C represent the uptake of C^{14} in the methyl group of the acetyl group in the chondroitin sulphuric acid as a function of time. Curves a, b, and c denote the corresponding uptake in the carboxyl group. The points D mark the retention in the methyl carbon in the acetyl group of the chondroitin sulphuric acid prepared from slices that had been boiled prior to incubation with the isotope.

It is seen from curves A, B, C, and a, b, c that in slices of calves' cartilage some methyl-labelled sodium acetate is built into the acetyl group of the chondroitin sulphuric acid, the labelled carbon being found both in the methyl group and, although to a lesser extent, in the carboxyl group. If the slices were boiled prior to addition of the isotope, there was no uptake (experiment D).

* Obtained from *The Radiochemical Centre Amersham, England.*

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Regulation of Urine Flow and Solute Excretion During Acute Acidosis Induced by Loading With Strong Mineral Acids in Hydropenic Dogs[1,2]

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WITH THE TECHNICAL ASSISTANCE OF Thomas I. Moxley, Clarice Drutz and Hilda S...

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PREVIOUS experiments have indicated that the urine flow of hydropenic dogs, after loading with acidic or basic salts, was dependent upon the number of osmotically active molecules in the urine, and independent of the degree of acidity or alkalinity of urine or plasma (1). Furthermore, the composition of urinary solutes after production of either acidosis (by NaH_2PO_4 infusion), or of alkalosis (by $NaHCO_3$ infusion) was the same in hydropenic as in overhydrated dogs. It appeared that tubular activities of acid base transfers on the one hand were independent of those of concentration—dilution processes on the other. Such data led to an investigation of the factors governing renal water conservation during acidosis.

The effects of acidosis on mechanisms of water and electrolyte conservation have been studied by many investigators under varying experimental conditions. Data of some indicated a diuretic effect of acidosis (2–6); those of others showed little or no change of urine flow (7–12). Many different mechanisms have been adduced to explain such variable effects of acidosis on the rate of urine flow. While Adolph (4) implicated the mobilization of anions for excretion as a mechanism producing diuresis during NH_4Cl ingestion, Gamble and others (3, 13) invoked cation loss as a prime factor. The claim that excretion of h... ions was of significance in the diuretic ... was supported by some (5, 6, 12, 14, ... denied by others (4, 11). High levels of ... sium ion excretion were thought to pl... in the genesis of the polyuria of diabet... However, maximal levels of diuresis ... tained in diabetic patients with but le... of potassium (17). Wolf (11) has des... effect of acid salts ($CaCl_2$) as ... implying that diuresis produced the... secondary to an increase of solute ... On the other hand, Sartorius and P... gested that the increase of urine volu... served during their experiment on loadi... phosphate buffer, was a phenomenon ... water diuresis (18). Folling (8) had p... a mechanism of 'dilution diuresis' to ex... effects of NH_4Cl. However, his data o... flow show little or no diuresis.

In these, and in other studies ... (19–25) differences of experimental co... prohibited a decisive analysis of data ... ing the effect of acidosis on urine flow. V... conditions included water intake, durat... testing, presence of disease states, and ... of different types of acids or acidifying s... induction of acidosis. Each factor, of ... would be expected to influence the ... excretion of water and solutes.

Present experiments were designed to ... the osmotic response of the kidney to a ... induced metabolic acidosis; and, if p... to eliminate any factor, which of itself ... tend to produce variations of urine flow.

Experimental control of urine flow ... preliminary periods was accomp... testing under conditions of st... hydropenia. The diuretic action of ac...

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[2] A preliminary report of these investigations has been published (*Federation Proc.* 12: 20, 1953).

Self

f Na, indistinguishable from that of e salts (1), was eliminated by the use e mineral acids as loading substances. nsatory mechanisms to be minimized d ammonia formation, and decreased n of sodium with attendant reduction of low (3, 7). Thus, the short-term nature nt experiments was expected to provide ons wherein the diuretic response to s would be at maximal levels.

METHODS

ardized conditions of hydropenia were ob- in a group of female mongrel dogs by with- f water for 20, and of food for 10 hours prior g (26, 27). On the morning of the experiment, tal, in doses of 25 mg/kg, was administered ously to produce anesthesia. Previously re- experiments indicated that Nembutal, under ns of hydropenic diuresis, had but little effect diuretic response (26). After anesthesia, a lene catheter was inserted into the external vein, and threaded into the right side of the This maneuver permitted intravenous injection entrated acid solutions at a site where dilution acid was maximal and immediate. At the same indwelling needle was placed into the femoral for purposes of blood sampling.

one or two preliminary periods of urine collec- fusion of acid was started via the intracardiac . Initial rates of infusion were 0.2–0.5 cc/min. 30-minute intervals the rate of infusion was d in stepwise fashion by increments of 0.5 until rates as high as 3–5 cc/min. were reached. xtremely high rates were usually followed by of the animal.

s were administered as the 0.6 M solution, a ation approximately four times that of plasma. g is a list of the acids employed: hydrochloric, lfuric, and phosphoric. Observations, made on d plasma, included those of urine flow, osmotic , pH, and concentrations of bicarbonate, de acidity, sodium, potassium, chloride, and te and urea. In some cases, clearances of r, mannitol, or inulin were determined before ring periods of acidosis. Creatinine was given aously in doses of 2 gm 1 hour prior to infusion Plasma levels usually achieved were between 20 mg %. Mannitol or inulin were given intra- y in amounts calculated to maintain constant levels of 20 mM/L., or of 100 mg %, respectively. cal techniques employed have been described re (27).

s suffered no apparent ill effect during infusion , according to the following experimental a) a normal level of blood pressure (measured nula in the carotid artery in four experiments) intained until 3–5 minutes prior to death. *b)* e values of creatinine, inulin, or mannitol ally maintained at normal levels throughout ation of infusions. Sharp declines of clearance values occurred a few minutes before death. *c)* The power of concentrating the urine remained at maximal levels throughout infusion of acid. This function was assessed from data on urine flow versus osmotic activity.

RESULTS

Flow Versus Load. Figure 1 presents a plot of the values of urinary flow versus osmotic load in 12 dogs rendered acidotic by infusion of strong inorganic acids. Data presented comprise 89 periods of urine collection. The solid line about which the data fall represents the typical flow load pattern noted previously in hydropenic dogs under conditions of osmotic diuresis produced by either mannitol loading or by injection of mercuhydrin (26). Gross inspection of the figure makes it evident that the pattern of flow-load data of acidotic dogs is closely similar to that of any non-specific type of osmotic diuresis (26, 28–30). Data for preliminary periods, not presented, showed that urine flows were minimal, 0.15–0.30 ml/min., and osmolarities were at maximal levels of 1300–1970 mOs/l. Such results confirmed those reported before on hydropenic dogs (26, 27, 30).

Urine flows in excess of 2.0 cc/min. were noted after acidosis induced by phosphoric acid, or after acidosis superimposed on mannitol diuresis. On the other hand, urine flows

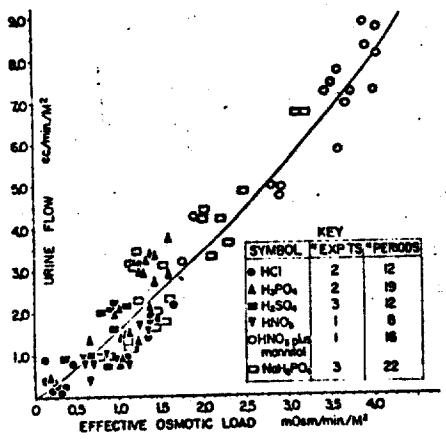

FIG. 1. Urine flow versus effective osmotic load. *Solid symbols* depict data of pure mineral acid loading. *Open symbols* depict data of combined mineral acid and mannitol, or of sodium dihydrogen phosphate loading.

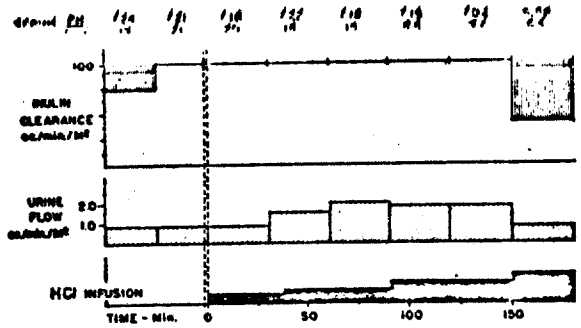

FIG. 2. Serum pH and CO_2 content, glomerular clearance, urine flow and infusion rate versus time before and after the development of severe acute acidosis. Values of serum pH and CO_2 content are designated by the printed numbers at the top of the graph. The values coincide in time with their abscissal distance from the zero ordinate.

rarely exceeded 1.5–2.0 cc/min. after loading with hydrochloric or with nitric acid alone. Why the buffer type H_3PO_4 would induce higher levels of urine flow than would the non-buffer acids is not entirely clear from these data. Nevertheless, compared to the high urine flows of 7–9 cc/min. observed after NaH_2PO_4 loading (1), the diuretic activity of acids employed herein was indeed somewhat small, and unrelated to the level of pH or CO_2 content of the serum or urine.

Figures 2 and 3 depict graphs of concurrent changes of serum pH and CO_2 content, glomerular clearance, and urine flow during induction of acidosis by infusion of HCl and HNO_3, respectively. Clearances of inulin or mannitol changed little, if at all while serum pH and CO_2 fell to values of 6.9 and 6.4 mM/l., respectively. Depression of glomerular clearance was noted during the terminal collection period in the two experiments of figures 2 and 3, and in three other experiments not shown in the figures. In other experiments terminal values for arterial pH and CO_2 content, even lower than those depicted, reached values of 6.42 and 2.2 mM/l., respectively. The level of arterial blood pressure, not shown in the present report showed no significant fall until 5 or 10 minutes before death, an observation in harmony with that reported elsewhere (31, 31a). Of interest is the observation that, both glomerular clearance and level of arterial blood pressure remained essentially constant while the pH of arterial serum was maintained between limits of 6.9 and 7.0 for periods of 1–2 hours.

Figures 2 and 3 also illustrate the small changes of urine flow occasioned by acute metabolic acidosis per se. After acidosis of HCl infusion, urine flow increased by 1.3 cc/min/m². After acidosis of HNO_3 infusion was imposed on a dog during mannitol diuresis, the flow increased by no more than 3.0 cc/min.

A correlation between urine flow and amount of solute injected and/or excreted may be invoked on the basis of the flow-load curve in hydropenic dogs (1, 26, 28)[3]. Injection of a given amount of a specific solute during osmotic diuresis with high flows would be expected to evoke a greater increase of urine flow, than that observed after injection of the same amount of this solute during preloading periods with low levels of urine flow. For example, infusion of HNO_3 during the preloading or resting hydropenic state resulted in a small increase of urine flow, from 0.4 to 0.6 cc/min. On the other hand, infusion of the same amount of HNO_3 during mannitol diuresis resulted in an increase of urine flow from 5.8 to 8.8 cc/min. It appeared that the increase of flow induced by acid infusion during hydropenia was a function of the rate of urine flow prior to the infusion. Such results had been expected on the basis of the flow-load relationship during osmotic diuresis of any type (1, 26, 28, 32).

Composition of Solutes in Urine and Plasma Before and After Induction of Acidosis. Table 1 presents values of solute concentration in the urine and serum of five representative dogs during selected periods before and after

[3] The plot of flow vs. load, shown in fig. 1 is a curve wherein the rate of increase of flow with respect to load increases in a direct manner with increasing loads. An exponential equation predicting flow on the basis of load has been derived from data in man (28).

of acidosis. Values in the row l 'preliminary' are averages of the riments shown. Values in the rows l 'HCl, HNO_3, etc.' have been se- m representative periods covering a ge of levels of serum pH and CO_2 n each case.

rum, during preliminary periods, ues of pH and CO_2 content were 7.32 mM/l. respectively. After infusion of m pH and CO_2 content fell to values s 6.88 and 6.41 mM/l. after HCl, 3.14 after HNO_3, 6.99 and 4.34 after d 6.42 and 2.20 mM/l. after H_3PO_4. f serum Na remained unchanged, or by 5–10 mEq/l. in most cases, an on in harmony with that reported during the acidosis of NH_4Cl in man rum K increased gradually with degrees of serum acidosis, reaching of high as 8.8 mEq/l. after HNO_3. f serum concentration of the loading ched were 137 mEq/l. in the case of for NO_3, and 24.2 mM/l. for PO_4.

e, during preliminary periods, values nd CO_2 content averaged 6.58 and M/l., respectively. Phosphate, the ffer, was present at a concentration of Concentrations of other urine solutes nilar to those reported previously n the hydropenic dog. During acidosis, urinary pH were as low as 4.64, while CO_2 content were 0.62 mM/l. (see r 'H_2SO_4' acidosis). Values of urinary varied from 93–255 mEq/l. after HNO_3; from 183–320 after H_2SO_4, and –190 mEq/l. after H_3PO_4. Values of n and of urea concentration were urine flow or load in a rough inverse a phenomenon observed by us under ifferent conditions of hydration and e equilibria (1, 27). Urinary chlorides values as high as 254 mEq/l. after l 274 after HNO_3; and as low as 5.4 after H_2SO_4 and 19 after H_3PO_4. Such patterns of urine chloride, after with univalent and multivalent anions, ected on the basis of other observa- renal anion antagonism (29), and on cretion (33).

s 4*A* and 4*B* depict a plot of values for osses of sodium and potassium versus effective osmotic load after infusion of acids. Data presented cover 51 periods of urine collection in eight experiments on eight acidotic dogs.

Sodium excretion reached values as high as 600–700 μEq/min. after infusion of acid. Over the small range of excreted solute load depicted, (0.2–2.0 mOs/min.), sodium constituted from 20–40% of the total urinary osmolarity, a fraction almost as high as that noted in dogs after mercuhydrin injection (26). After infusion of large amounts of sulfuric acid, values of sodium excretion fell significantly to the left of most of the data. Such deviations are probably due to the valency and to the activity coefficient of the sulfate ion. Similar deviations of sodium data, noted after phosphoric acid, were not so great as those noted after sulfuric acid.

Figure 4*B* shows a graph of values of potassium excretion versus effective osmotic load. Data presented cover the same periods of urine collection as does figure 4*A*. It is apparent that potassium, excreted at a rate slower than that of sodium, did not reach levels of minute loss greater than 200 μEq/min. Of interest was the observation that potassium excretion increased along with the increasing of acidity of serum and urine.

Potassium accounted for about 10–20% of the total urinary solutes over the range of loads, (0.2–2.0 mOs/min.) observed herein. This fraction was similar to that noted after loading with acidifying, alkalinizing and neutral salts of sodium (1–3).

Figure 5 presents data for excretion rates of

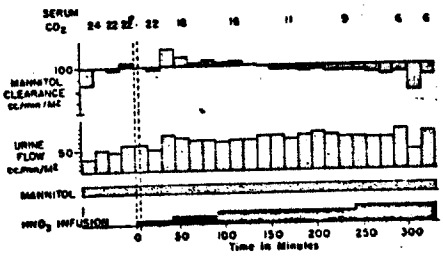

FIG. 3. Serum CO_2 content, glomerular clearance, urine flow, and infusion rates during mannitol diuresis both before and after HNO_3 infusion. Values of serum CO_2 content are designated in a manner identical to that in fig. 2.

TABLE 1. COMPOSITION OF SOLUTES IN URINE AND PLASMA DURING SELECTED PERIODS OF INORGANIC ACID ACIDOSIS IN FIVE HYDROPENIC DOGS

Period	Concurrent Time	Urine Flow	pH	CO_2	Cl	PO_4	Na	K	Ca	Urea	Osmotic Activity
	min.	cc/min/m²	mM/l.						mEq/l.	mM/l.	mOs/l.
Prelim.											
Urine		0.56	6.58	3.32	261	16	216	69	6.4	671	1400
Plasma			7.32	24.5	112	1.7	142	4.36	5.3	6.14	
HCl											
U-2	61–89	2.17	6.21	0.62	248	20.9	230	28		185	750
P			7.18	14.9	127		146	4.58	4.90	8.00	
U-3	89–120	1.81	5.99	0.62	254	20.9	241	51.5		118	
P			2.16	8.60	126		146	5.00	5.10		
U-5	135–150	0.78	5.98	0.88	189	13.8	158	56		110	
P			6.88	6.41	137		158	5.92	5.20	7.07	
						NO_3 mM/l.					
HNO_3											
U-2	59–90	0.88			274	50.8	255	72.0		420	1226
P			7.35	16.6	110	5.04	137	4.50		4.22	
U-6	180–214	0.81			126	45.9	145	94.0		190	763
P			7.03	13.5	108	9.51	131	4.30		4.43	
U-10	318–337	0.20			83.6	50.0	92.5	85.0			
P			6.53	3.14	96	15.1	124	8.80			
H_2SO_4											
U-1	34–63	2.14	4.64	0.62	5.40		290	42.5	13.5		58
P			7.18	12.3	106		148	4.00	4.60		
U-2	63–92	2.23	4.78	0.65	5.84		320	28.0	12.3		421
P			6.99	4.34	104						
H_2SO_4											
U-3	30–59	1.71			33.2		198	55	4.30	222	827
P				15.2	106		129	4.00	4.78	7.75	
U-4	59–76	0.98		2.69	15.0		183	83.5	3.10	155	660
P				13.1	106		130	4.15	4.45	9.05	
H_3PO_4											
U-2	30–41	2.76	6.27	4.42	134	72.4	190	30.0	3.05		
P			7.23	20.7	116	3.80	136	3.64	5.50		
U-5	82–96	2.90	5.92	2.76	37.3	134	157	28.0	3.40		
P			7.08	14.9	117	7.10	130	3.90	6.45		
U-9	135–146	3.85	5.39	1.47	24.9	164	140	28.0	3.20		
P			6.87	7.60		14.1	135	4.32			
U-13	189–206	2.86	4.99	0.95	19.0	187	145	32.0	3.50		
P			6.42	2.20	106	24.2	133	5.60			

potassium ion and of titratable acid during induction of acidosis with phosphoric acid. The parallelism of excretory pattern of the two constituents is obvious on gross inspection of the figure. This is not an isolated observation of the present report, similar patterns having been observed in other loading experiments with phosphoric acid, sodium dihydrogen phosphate and potassium acid phosphate (34). After non-buffer acids were given, potassium output increased while urinary pH and CO_2 content decreased. Such changes are difficult to explain on the basis of an assumed reciprocal exchange mechanism for potassium and hydrogen ions across tubular membranes (35, 36).

Data on minute losses of anion, apparent by calculation of values in table 1, were closely similar to those reported elsewhere (27, 29), after loading with sodium salts of chloride, nitrate, sulfate and phosphate.

Changes of Clearance During Development of Acidosis. Calculations of glomerular clearance were made from determinations of creatinine, inulin, or mannitol in six experiments comprising 64 periods of observation. Glomerular clearance remained unchanged until the last one to two periods in two dogs; increased and then declined gradually in two dogs; and declined progressively in two dogs. Calculations of urea clearance were made in

wo other experiments
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ained unchanged until
n both instances.

In general, glomerular
levels of
CO_2 content of serum
Where clearances
calculated value was
min/m².
Where clearance
values were high, 130–
gradually fell to levels of
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has been observed recently
(1) and elsewhere (37).

DISCUSSI

Present data indicate
response to mineral acid i
predominantly on the nu
active molecules (effec
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non-electrolytes, neutral,
salts (1, 28); or by inje
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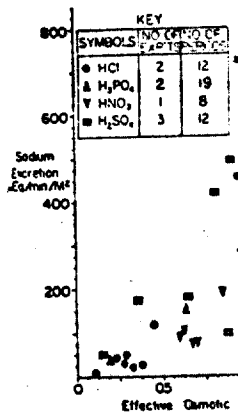

FIG. 4A. Sodium excretion
load.

other experiments covering 22 additional ls of observation. Urea clearance re- ned unchanged until the last two periods oth instances.

general, glomerular clearance did not fall levels of 50 cc/min/m², or less until the content of serum was less than 10 mM/l. re clearances remained constant, the ulated value was around 100–110 cc/ m². Where clearances declined, initial es were high, 130–160 cc/min/m², then dually fell to levels of 80–100 cc/min., and lly to values as low as 20–30. Unchanging rances during metabolic acidosis in dogs been observed recently in this laboratory[2] and elsewhere (37).

DISCUSSION

resent data indicate that the diuretic onse to mineral acid loading is dependent lominantly on the number of osmotically ve molecules (effective osmotic load) ming excretion. Such a response is charac- stic of an osmotic diuresis, forced by load- with a wide variety of solutes, i.e. with electrolytes, neutral, acidic or alkaline s (1, 28); or by injection of organic mer- al compounds (26). That the osmotic resis of acid loading is independent of the ree of serum acidosis is apparent from a parison of present data with those noted a loading with sodium acid phosphate. r phosphoric acid, with serum pH and CO_2 ent as low as 6.8 and 4.0 mM/l., respectively, urine flow reached maximal levels of 3–4 cc/min. On the other hand, after NaH_2PO_4, with serum CO_2 content no less than 15 mM/l., urine flow reached levels of 6–8 cc/min. (1). Such findings had been expected on the basis of previous work showing an identity of flow-load response during extreme degrees of acidosis and of alkalosis.

In general, urine flow during severe acidosis was not so high as that noted in other forms of osmotic diuresis (26–29). It is difficult to explain such a response on the basis of a reduced glomerular filtration in view of observations on normal clearance values reported here and elsewhere (1, 37). One might explain low levels of urine flow after mineral acid acidosis by an analogy to the diuresis noted after loading with sodium salts of anions corresponding to the various mineral acids—e.g. NaCl, $NaNO_3$, Na_2SO_4 and NaH_2PO_4. Since the diuresis following sodium salts is primarily osmotic in origin, one might predict a markedly reduced diuretic response to loading with the pure mineral acids corresponding, in each case to one of the aforementioned sodium salts. This prediction is based on the fact that pure acids do not produce the increase of plasma osmolarity induced by their sodium salts.

Differences of urine flow with similar degrees of serum acidosis were noted after loading with various acids. Such variable diuretic responses may be explained on the basis of the mode of excretion of the loading anions perfusing the kidneys. Thus phosphates, excreted at a level close to that of glomerular clearance (1, 34) evoked the highest urine flows observed in the present study. Sulfates, as anticipated (30, 33, 38) also produced a

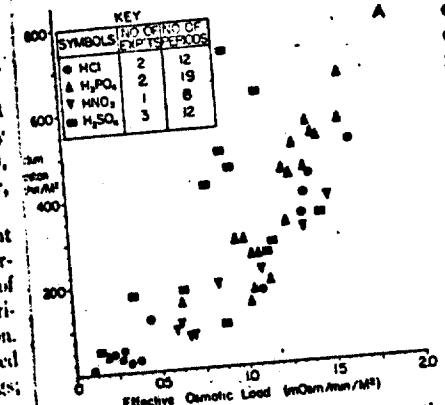

FIG. 4A. Sodium excretion versus effective osmotic load.

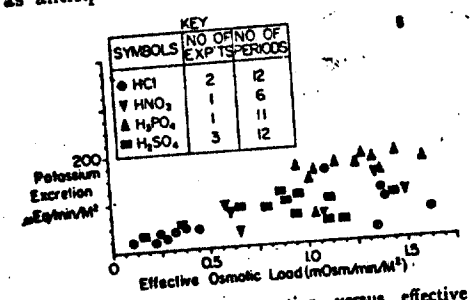

FIG. 4B. Potassium excretion versus effective osmotic load.

relatively high urine volume. On the other hand, chlorides and nitrates, presumably reabsorbed in significant amounts, do not induce so vigorous a diuresis as do sulfates and phosphates. One may infer that the greater the amount of anion reaching the final urine, the greater the final solute load excreted and, consequently, the greater the urine flow.

It is difficult to explain data reported herein by the hypothesis that acidosis suppresses antidiuretic activity and thereby increases urine flow (18). Objections to this hypothesis are as follows: *a*) results on urine flow and osmolarity in our acidotic dogs were characteristic of the hydropenic state, wherein antidiuretic secretion presumably is at maximal levels (39–41). *b*) Factors diluting body fluids (e.g. latter stages of water diuresis, intraperitoneal or subcutaneous injection of 5% glucose) induced antidiuretic responses (42–46). Since infusion of acid into a slightly alkaline extracellular fluid tends to reduce osmotic activity, one would expect increased rather than decreased antidiuretic activity.

A tentative analysis on the mechanism of changes in water and electrolyte excretion during acidosis is as follows: *a*) reduction of serum pH or CO_2 content per se has little if any effect on the water and electrolyte function of the kidney. *b*) However, the increased concentration of loading anion in the plasma (and in the glomerular filtrate) will induce a

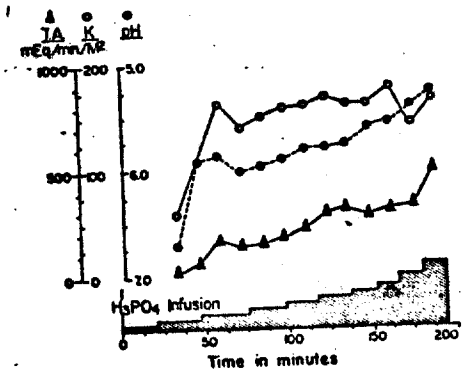

FIG. 5. Minute losses of titratable acid and of potassium, and pH of urine versus concurrent time during development of acidosis induced by phosphoric acid loading. Relative rate of infusion of acid is depicted by the shaded bar along the base-line.

change of active anion transport across tubular cells, the net result of which would be increased urinary excretion of anions. Cation excretion would increase passively to satisfy the requirement of electroneutrality. *c*) The increased a[illegible] of anion and cation issuing from a [illegible]ular site would be presented [illegible]bule which responds osmometrically, [illegible] reabsorbing water, the volume of which is governed by the quantity of solute in the lumen. *d*) Quantity of solute presented to distal tubule, and hence, appearing in final urine is dependent on the type of acid employed as a loading agent. This dependence is such that chloride excretion follows a pattern similar to that of the Hofmeister series as observed previously during the osmotic diuresis of various sodium salts (27, 29, 30).

How acidosis per se could alter a tubular mechanism of active anion transport is uncertain. It is difficult to conceive of any significant change of net pore charge in tubular membranes with the relatively small changes of plasma pH in acidotic animals. Furthermore, an hypothesis of fixed pore charge (47) would require increased permeability to anions if the pH of fluid bathing tubular cells is reduced sufficiently. Such increased permeability of tubular membranes, together with the increased anion excretion observed herein, would imply tubular secretion of the anions. On the other hand, increased plasma concentration of a given anion, induced by acid loading may alter tubular reabsorptive transport by virtue of changing the relative mobility and transference numbers of ions in the glomerular filtrate. Available evidence does not permit a precise analysis of the magnitude and direction of ion transport systems in tubular cells. Obviously, the testing of such hypotheses of active transport (secretory or reabsorptive) would require experimental techniques other than those employed in the present study.

SUMMARY

Severe acidosis was induced in nembutalized dogs by intracardiac infusion of 0.6 M solutions of hydrochloric, nitric, phosphoric, and sulfuric acids. In some experiments mannitol plus nitric acid was injected; in others 20% NaH_2PO_4 was injected. Animals had been

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SUMMARY

s was induced in nembutalize
rdiac infusion of 0.6 M solution
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some experiments mannit
was injected; in others 20
injected. Animals had been
ived of water for 20 hours and food for
hours preceding experiments and were
eting urine at flows of 0.15–0.30 ml/min.
osmolarities of 1300–1970 in mOs/l.
After loading, serum pH and CO_2 content
to values as low as 6.42 and 2.2 mM/l.,
ectively. Levels of loading anions in
ma amounted to increments of 15–25
l. Urine flow increased to no more than
cc/min. after loading with HCl and HNO_3,
to 2–4 cc/min. after loading with H_3PO_4
H_2SO_4. Urine flow increased by 3.0 cc/min.
n nitric acid loading was superimposed
mannitol diuresis. Sodium acid phosphate
ing resulted in high urine flows of 7–9 cc/
. All values of urine flow versus solute
fell closely around a plot of similar values
rved previously in hydropenic diuresis
ced by many different loading solutes.
ppeared that urine flow and solute excre-
of hydropenic dogs were independent of
pH and CO_2 content of plasma or urine.
ariations of urine flow during mineral acid
sion could be explained mainly on the na-
of excretion of the loading anion. Thus, the
ive diuretic effect of the anions was of the
er, $SO_4^{=} > H_2PO_4^- > NO_3^- > Cl^-$. A possible
ence of such anions on changes of anion
sport was adduced. The role of filtration
me, antidiuretic secretion and of cation
sport in the renal response to acidosis was
ussed.

With respect to electrolyte excretion, urinary
um loss reached levels as high as 600–700
min. with total solute losses as low as
-2.0 mOs/min. Sodium excretion per unit
l solute during sulfuric acid loading was
her than that noted during the other types
ineral acid loading. Chloride losses reached
es of 400–600 μEq/min. during HCl and
NO_3 infusion, but rarely exceeded 100 μEq
ing H_3PO_4 and H_2SO_4. Excretion of potas-
m increased after all types of acidosis,
aining levels of 150–200 μEq/min., or no
re than one-fourth to one-third that of so-
m. Concomitant excretion rates of potas-
m and titratable acid increased in a parallel
nner during the progressive acidosis of
sphoric acid loading. The implication of
s finding to a postulated potassium ion–hy-
gen ion exchange mechanism was discussed.
It was concluded that serum acidosis per se
has little if any effect on the rate of urine flow
during hydropenia. Moreover, observed in-
creases of urine flow during acidosis could be
explained mainly on the basis of primary
changes of anion excretion mechanisms in tubu-
lar cells.

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C. THE EFFECTS OF THE INJECTION OF CERTAIN SALTS ON THE CALCIUM, MAGNESIUM AND INORGANIC RHOSPHORUS OF THE SERUM OF THE RABBIT.

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(*Received March 1st, 1934.*)

BROOKFIELD [1933] showed that in the serum of the rabbit there was an inverse relationship between calcium and magnesium, a direct relationship between magnesium and inorganic phosphorus and an inverse relationship between calcium and inorganic phosphorus, the latter of which had been previously observed by Dupré and Semeonoff [1931] and Bourne and Campbell [1932]. The rises and falls in the concentrations of the three ions in the serum were correlated with their relative amounts in the diet. The relationships seemed worthy of further study under other experimental conditions, in order to determine whether the effects observed with diets varying in their mineral contents were of more general significance, and accordingly another series of experiments has been carried out.

Solutions of salts containing Ca^{++}, Mg^{++} and $PO^{\equiv}_4$ ions have been injected subcutaneously into rabbits and their effects on the calcium, magnesium and inorganic phosphorus of the serum observed.

EXPERIMENTAL.

Experiments were made on fully grown male rabbits maintained on a diet of cabbage and oats, which (with one exception) had fasted for about 18 hours. Calcium gluconate, laevulate and lactate, magnesium sulphate and lactate and disodium hydrogen phosphate were the salts employed, while control experiments were carried out with sodium chloride and sodium sulphate. The salts, dissolved in the minimum volume of water, were injected subcutaneously into the flank. 5 ml. of blood were withdrawn from the lateral vessel of the ear immediately before the injection and on three separate occasions afterwards. The effects were observed over periods varying from 1½ to 6 hours. The methods of estimating calcium, magnesium and inorganic phosphorus were the same as those used in the dietary experiments [Brookfield, 1933].

The influence of two factors called for control before the results of the experiments could be interpreted; firstly, the effects of four small haemorrhages recurring at relatively short intervals and secondly, the effects of the injection of a solution of an indifferent salt.

1. *The effects of bleeding.* (*a*) *Successive small haemorrhages.* 5 ml. of blood were removed from a rabbit four times at half-hourly intervals; 9 days later the same procedure was repeated, but at hourly intervals and after a further 22 days the animal was bled again, this time at 2-hourly intervals. In the

Phos + Sulf

first experiment the haemoglobin percentage was unaltered at the fourth bleeding: in the other two experiments a fall of 8 % was noted. The serum-calcium, magnesium and inorganic phosphorus values are recorded in Table I.

Table I. *Serum-calcium, magnesium and inorganic phosphorus. Small haemorrhages at short intervals.*

Rabbit 20. Weight 2010 g. 4 bleedings of 5 ml. on each occasion.

Calcium mg./100 ml.	Magnesium mg./100 ml.	Inorganic phosphorus mg./100 ml.
½-hourly intervals		
16·55	1·652	3·152
15·70	1·694	2·626
15·15	1·597	2·551
14·95	1·590	2·716
1-hourly intervals		
13·29	2·886	4·382
13·29	2·886	4·382
12·94	2·794	4·762
12·72	2·749	4·794
2-hourly intervals		
13·53	3·615	3·750
13·53	3·542	3·936
13·90	3·357	4·521
13·67	3·204	4·654

It will be seen that the magnesium value tends to fall, but at a very slow rate. The calcium figures show variable results, depending on the time interval. The four bleedings at half-hourly intervals have caused a fall of 1·6 mg. (10 %). Hourly bleedings cause a slighter fall (4 %) and 2-hourly bleedings none. A fall in the inorganic phosphorus followed by a rise after one hour results from bleeding at intervals of half an hour, while with hourly or 2-hourly bleedings the inorganic phosphorus rises.

(*b*) *Progressive changes during a single haemorrhage.* The rapidity with which the fall in the serum-calcium takes place is illustrated by the results recorded in Table II.

Table II. *Serum-calcium and magnesium values during bleeding. Blood taken in two samples of 5 ml. each.*

Rabbit no.	Time of bleeding	Calcium mg./100 ml.	Magnesium mg./100 ml.
19	11.45–12.15	15·28	1·722
	12.15–12.45	14·44	1·694
21	4.30–4.45	15·73	1·893
	4.45–5.0	15·35	1·886
19	10.45–11.0	12·60	1·907
	11.0–11.10	12·25	1·896
20	11.15–11.25	14·35	1·963
	11.25–11.30	14·25	1·951

Each animal was submitted to a single haemorrhage and the blood collected in two successive 5 ml. samples. In one experiment where the time of bleeding was rather prolonged, a fall of 0·8 mg. (6 %) was noted. The effects of haemorrhages on the serum-calcium of rabbits have been referred to by several writers. A decrease of 10 % or more has been reported when observations have been made

at a short interval after the haemorrhage by Stransky [1915], Clark [1920], Stewart and Percival [1927] and Culhane [1927]. Seven hours after bleeding, Moritz [1925] found a slight and inconstant decrease in the serum-calcium value. A fall in the magnesium also was noted by Stransky. These results show that the variations in the concentrations of calcium, magnesium and inorganic phosphorus caused by bleeding are so slight as to have little or no significance in the interpretation of the findings in the injection experiments, which will now be discussed.

2. *The effects of the injection of an indifferent salt.* Sodium chloride was injected in an amount somewhat in excess of those of the other salts employed. The results are recorded in Table III.

Table III. *Injection of sodium chloride. Serum-calcium, magnesium and inorganic phosphorus values.*

Rabbit 24. Weight 2300 g. 5 ml. bleedings. Fasting.

Time after injection (hours)	Calcium mg./100 ml.	Magnesium mg./100 ml.	Inorganic phosphorus mg./100 ml.
0	13·29	2·786	3·820
0·30 g. NaCl (0·0062 g. equivalent per kg. in 9 ml. water injected subcutaneously).			
½	13·29	2·806	4·154
1	12·67	2·726	3·434
4	13·06	2·557	3·701

There is a slight fall in all three constituents after one hour with a tendency on the part of the calcium and inorganic phosphorus to return to their initial values after 4 hours. These changes are unimportant in comparison with the results which follow, and they show that probably any effect observed in the injection of salts containing Ca^{++}, Mg^{++}, or $PO^{\equiv}_4$ was to be attributed to these ions themselves.

3. *The injection of solutions containing the Ca^{++}, Mg^{++}, $PO^{\equiv}_4$ and $SO^{=}_4$ ions.* An attempt was made, in each case, to introduce an amount of salt sufficient to cause a marked effect on the several blood-constituents under review without producing untoward symptoms. The success achieved was only partial. No symptoms were noted after the injection of the calcium salts. Slight ataxia of the hind-limbs resulted from the injection of magnesium sulphate and lactate. Definitely toxic effects were observed however with disodium phosphate and sodium sulphate. In both cases there was some degree of collapse after the injection. The animal injected with disodium phosphate showed marked muscular twitching but ultimately recovered. The animal which received sodium sulphate was found dead 2 days later. No urine was passed during the period of any of the experiments except by the rabbit which received sodium sulphate. None of the animals suffered from diarrhoea.

Results.

For purposes of comparison the amounts of the salts have been expressed as g. equivalents per kg. body weight. The results of the injections of the calcium salts are set out in Table IV, of the magnesium salts in Table V, of sodium sulphate in Table VI and of disodium phosphate in Table VII.

1. *The interrelations of calcium and magnesium.* In each of the 4 experiments where calcium salts have been injected approximately equivalent amounts of calcium have been given. A moderate increase in the serum-calcium has been the result amounting on the average to 3·0 mg. or 23 % of the initial value.

The rise to the maximum takes place within one hour and is followed by an almost equally rapid fall. The rise in the serum-calcium is accompanied by a progressive fall in the serum-magnesium. In the case of the injection of calcium gluconate, there may be a slow return towards normal at 6 hours. The average fall amounts to 1·0 mg. or 37 % of the initial value though it should be noted that in 3 of the experiments (Table IV) the fall appears still to be continuing at the time of the final observation.

Table IV. *Injection of calcium salts. Serum-calcium, magnesium and inorganic phosphorus values.*

	Salt injected	Time after injection (hours)	Calcium mg./100 ml.	Magnesium mg./100 ml.	Inorganic phosphorus mg./100 ml.
Rabbit 19. 2520 g. Fasting	Gluconate (0·6 g. ≡ 0·0028 g. equiv. Ca/kg.)	0	13·17	2·389	3·720
		½	16·13	1·896	3·861
		2	14·91	1·590	4·106
		6	14·32	1·748	4·152
Rabbit 23. 2000 g. Fasting	Gluconate (0·75 g. ≡ 0·0035 g. equiv. Ca/kg.)	0	13·29	3·091	4·826
		½	16·39	2·567	4·700
		1	16·75	2·097	4·609
		1½	17·77	1·896	4·700
Rabbit 23. 2200 g. Fasting	Laevulate (0·6 g. ≡ 0·0039 g. equiv. Ca/kg.)	0	12·27	3·044	4·252
		½	15·10	2·635	3·990
		1½	14·57	2·136	—
		2½	13·48	1·860	—
Rabbit 23. 2170 g. Fasting	Lactate (0·46 g. ≡ 0·003 g. equiv. Ca/kg.)	0	14·46	2·332	2·977
		½	17·02	1·992	3·106
		1	14·97	1·620	2·927
		2	14·82	1·544	3·247

Though the magnesium salts have been injected in amounts equivalent to those of calcium the effects have been considerably greater. The rise in the serum-magnesium averages 5·6 mg. over the initial value, as compared with a rise of 3·0 mg. in the calcium value caused by injections of corresponding amounts of calcium salts. This rise takes place rapidly but is more sustained than in the case of calcium, and it is clear that either the rate of absorption of magnesium salts is much less rapid than that of calcium salts, or alternatively and more probably

Table V. *Injection of magnesium salts. Serum-calcium, magnesium and inorganic phosphorus values.*

	Salt injected	Time after injection (hours)	Calcium mg./100 ml.	Magnesium mg./100 ml.	Inorganic phosphorus mg./100 ml.
Rabbit 19. 2680 g. Fed with cabbage and oats	Sulphate (0·37 g. ≡ 0·003 g. equiv. Mg/kg.)	0	14·69	2·109	2·779
		¾	11·32	8·078	2·080
		1½	10·74	8·258	1·890
		4¾	13·31	3·031	3·086
Rabbit 25. 2750 g. Fasting	Lactate (0·37 g. ≡ 0·0031 g. equiv. Mg/kg.)	0	12·62	2·318	3·682
		¾	11·85	6·726	3·262
		1¾	10·54	7·066	3·147
		5	10·61	5·833	3·608

that the removal of excess magnesium from blood is much less readily accomplished. Where magnesium sulphate has been injected (Table V) the serum-magnesium is approaching the normal value after 5 hours, but at the same interval after the injection of the equivalent amount of magnesium lactate (Table V) the serum-magnesium is nearly 3 times greater than the initial value.

These increases in the magnesium concentration have resulted in a marked fall in the calcium value in every experiment. This fall in calcium has been more rapid and greater in extent (4·0 mg. or 27 % of the initial value), following the injection of magnesium sulphate (Table V). The calcium value in this experiment is approaching the initial level after 6 hours. Following the injection of magnesium lactate the serum-magnesium continues at a high level for a considerable time and there is a correspondingly prolonged depression of the serum-calcium, which after 5 hours is still at the low level of 10·6 mg.

The inverse relationship found to exist between calcium and magnesium in the dietary experiments is therefore borne out by the results of these subcutaneous injections of calcium and magnesium salts.

Previous observations of the effects of the injection of calcium and magnesium salts on the calcium and magnesium content of serum are fragmentary and in some cases conflicting. Salvesen *et al.* [1924, 2] found that the magnesium content of the blood of dogs was depressed by the intravenous injection of calcium chloride. Stransky [1915] and Meneghetti [1927] observed a fall in the serum-calcium of rabbits after the injection of magnesium sulphate, the latter worker also showing that the diffusible calcium was increased. A number of observers have shown that the injection of magnesium salts leads to an increase in the calcium excretion and *vice versa*. Thus Mendel and Benedict [1909] found that the injection of calcium chloride into dogs and rabbits led to loss of magnesium, while the injection of magnesium sulphate and chloride increased the excretion of calcium. Greenwald and Gross [1925] noted a temporary increase in the excretion of magnesium in dogs injected with calcium chloride. After introducing metallic magnesium under the skin of rabbits, Reding and Slosse [1923] noted a considerable increase in the output of calcium, and loss of calcium was also observed by Schiff [1920] and by Schiff and Stransky [1920] in infants injected with magnesium sulphate. While a fall in the serum-calcium, accompanied by an increased excretion of calcium, was noted by Pribl [1929] in rabbits after the injection of magnesium sulphate, a rise in the serum-calcium value with calcium retention following the injection of magnesium lactate was reported. Condorelli's [1926] findings are also at variance with those recorded above, in that he observed a considerable elevation of the serum-magnesium following the injection of calcium lactate into rabbits.

2. *The effects of the sulphate ion.* One possible complicating effect in the experiment with magnesium sulphate is the presence of the sulphate ion, for the fall in the calcium value was less when magnesium was introduced as the lactate. Since the injection of phosphate depresses the serum-calcium it was possible that the injection of sulphate might have had a similar effect, and this was controlled in an experiment in which sodium sulphate was injected in amount equivalent to twice that of the magnesium sulphate used. Table VI shows that this has

Table VI. *Injection of sodium sulphate (1·0 g. Na_2SO_4, $10H_2O \equiv 0·006$ g. equiv. Na/kg.). Serum-calcium, magnesium and inorganic phosphorus values.*

Rabbit 23. Weight 2500 g. Fasting.

Time after injection (hours)	Calcium mg./100 ml.	Magnesium mg./100 ml.	Inorganic phosphorus mg./100 ml.
0	14·34	2·629	4·700
½	13·52	2·629	3·536
1½	12·20	2·505	3·106
4	11·20	2·357	3·572

resulted in a considerable fall in the serum-calcium but one different in character from that caused by the injection of magnesium sulphate. The fall is much more gradual, and although still continuing at the time of the last observation, 4 hours after the injection, is less than that produced by half the equivalent amount of magnesium sulphate (Table V). It is of interest that Stransky [1915] found a much increased calcium excretion after the injection of sodium sulphate into a rabbit. Table VI shows that while sodium sulphate has no effect on the serum-magnesium, the serum-inorganic phosphorus is depressed by 1·6 mg. (34 %) to rise again after one and three quarter hours, when it moves in a direction opposite to that of calcium.

3. *The interrelations of calcium and inorganic phosphorus.* The changes in the serum-inorganic phosphorus after the injection of the three calcium salts (Table IV) are so slight that it appears that injected calcium has no direct effect on the inorganic phosphorus. When, however, disodium phosphate is injected there is an immediate fall in the serum-calcium (Table VII). In this experiment the phos-

Table VII. *Injection of disodium hydrogen phosphate (0·57 g. $Na_2HPO_4, 12H_2O \equiv$ 0·0047 g. equiv. P/kg.). Serum-calcium, magnesium and inorganic phosphorus values.*

Rabbit 19. Weight 2300 g. 5 ml. bleedings. Fasting.

Time after injection (hours)	Calcium mg./100 ml.	Magnesium mg./100 ml.	Inorganic phosphorus mg./100 ml.
0	12·97	2·520	4·121
1	11·39	2·308	10·01
2	10·74	2·423	10·51

phate was injected in amount equivalent to 1·5 times the amounts of the salts used for the calcium injections. The rise in the serum-inorganic phosphorus was rapid and relatively prolonged: at the end of one hour it was 5·9 mg. (140 %) above the initial value, and at 2 hours was at a slightly higher level. At the same interval after the injection, the serum-calcium had fallen by 2·2 mg. or 17 %. Samples were unobtainable after 2 hours owing to the collapsed state of the animal.

From these results it is plain that the inverse relationship between the calcium and inorganic phosphorus of serum observed in the dietary experiments is only partially maintained when calcium or phosphate is injected. The results of the injection of phosphate are in accord with the dietary experiments inasmuch as the serum-calcium falls as the inorganic phosphorus rises. Several previous workers have reported a depression of the serum-calcium on injecting phosphate. Ringer [1917] produced a fall of the serum-calcium to 6 mg./100 ml. with the development of tetany by injecting dogs intravenously with disodium phosphate. Tisdall [1922] repeated his experiments with a similar effect, while Salvesen *et al.* [1924, 1] obtained the same results when phosphates were administered orally. The calcium thus displaced from the serum is apparently largely excreted since Greenwald and Gross [1925] found a rise in the calcium excretion in dogs after the injection of neutral sodium phosphate, while Boyd *et al.* [1930] observed, in addition to a fall in the serum-calcium, an increased urinary excretion of calcium after injecting sodium glycerophosphate into the same animals.

In the present investigation injection of calcium has produced no definite fall in the serum-inorganic phosphorus, so that in this respect there is no effect analogous to the inverse relationship of the dietary experiments. The results of other workers are somewhat variable. Bomskov [1930] found that a fall in the

inorganic phosphorus followed the injection of calcium gluconate into rabbits. Salvesen *et al.* [1924, 2] and Collip [1926], using dogs, reported a small rise in the inorganic phosphorus value on injecting calcium chloride. Condorelli [1926] found a greater rise in the rabbit injected with calcium lactate. Greville [1931] found the inorganic phosphorus of the serum of two rabbits slightly elevated after the intravenous injection of calcium laevulate, while Apitzsch [1931] observed that the serum-inorganic phosphorus of human subjects was raised after the oral administration of calcium lactate. Thus the balance of evidence suggests that there is no disappearance of phosphorus from the blood, though it is noteworthy that Greenwald and Gross [1923] found an increased excretion of phosphorus in the dog after the injection of calcium chloride.

4. *The interrelations of magnesium and inorganic phosphorus.* Following the injection of magnesium (Table V) there is a fall in the serum-inorganic phosphorus amounting, after the injection of the sulphate to 0·9 mg. (32 %) and after that of the lactate to 0·5 mg. (15 %). The somewhat greater effect of magnesium sulphate is possibly accounted for by the synergic action of the sulphate ion which itself lowers the serum-inorganic phosphorus (Table VI). It will be seen that the injection of phosphate (Table VII) caused no appreciable change in the serum-magnesium, just as the injection of calcium caused no effect on the serum-inorganic phosphorus. These results are not in accord with the direct relationship between magnesium and inorganic phosphorus observed in the dietary experiments.

Discussion.

The injection experiments differ from the earlier dietary experiments in two important respects which may account to some extent for some of the divergences in the results of the two series. Firstly, whereas in the injection experiments salts were introduced subcutaneously and rapidly absorbed into the circulation, in the dietary experiments much smaller amounts of the relevant ion were slowly absorbed from the intestine over a period occupying 2 hours or more. Secondly, while two ions only were concerned in the injection experiments, a number of ions were being absorbed simultaneously in the dietary experiments, as well as other constituents which may themselves exercise important effects.

These differences do not appear to be of sufficient importance materially to affect the relationship existing between the calcium and magnesium of serum, which in both series of experiments is inverse. It is noteworthy that the effects of the injections have not been as drastic as might be anticipated. In the dietary experiments the rises and falls in the serum-calcium corresponded with similar rises and falls in the serum-magnesium, whereas in the injection experiments the greater and more rapid increase in the concentration of the one ion in the blood has produced a fall in the other hardly greater than those seen in strictly physiological conditions. It is evident that there exist mechanisms whereby the organism can resist attempts to lower a blood constituent to an undesirable level.

When the relations of calcium and magnesium to inorganic phosphorus are studied, differences in the results of the two series of experiments are at once apparent. Although the injection of phosphate causes a fall in the serum-calcium the converse is not observed, since when a calcium salt is injected, the serum-inorganic phosphorus is virtually unaffected. The inverse relationship between the serum-calcium and inorganic phosphorus as observed in the dietary experiments therefore appears to operate in one direction only. The direct relationship between magnesium and inorganic phosphorus observed in the dietary experiments receives no support from the results of the injections, since while the

injection of phosphate leaves the serum-magnesium unaffected, the injection of magnesium salts depresses the inorganic phosphorus so that the two constituents are actually in inverse relationship.

The lack of reciprocity between calcium and inorganic phosphorus on the one hand and between inorganic phosphorus and magnesium on the other which is indicated by the results of the injection experiments appears to find an explanation when the relative concentrations of these constituents in the blood are considered. Normally the calcium of serum is more than equivalent to the inorganic phosphorus, so that it might be anticipated that, when more phosphate was introduced into the blood-stream, combination with calcium would occur with the deposition in some suitable site of calcium phosphate and a consequent fall in the serum-calcium value. Since calcium is present in excess of inorganic phosphorus, it is hardly surprising that the introduction of further calcium produces no fall in the inorganic phosphorus. Similarly, the inorganic phosphorus of serum is more than equivalent to the magnesium, so that, when more magnesium is introduced, it seems reasonable to expect that some of the magnesium will combine with inorganic phosphorus, the disappearance of which from the blood will lead to a fall in the serum-inorganic phosphorus. On the other hand, the addition of further phosphate when inorganic phosphorus is already in excess need hardly be expected to cause a fall in the serum-magnesium.

The injection experiments suggest that fluctuation of the serum-inorganic phosphorus is the chief controlling factor in the calcium-inorganic phosphorus relationship observed in the dietary experiments. This is in harmony with the explanation suggested by Fraser [1932] who regards the relationship as dependent on the periodical liberation of calcium phosphate from the bones. The calcium plays a passive rôle, in that calcium deficiency causes calcium phosphate to leave the depots with consequent rise in the serum-inorganic phosphorus value, whereas the presence of a sufficiency of calcium in the blood leads to a discontinuance of the process, so that the serum-inorganic phosphorus tends to fall. It is obvious that the depots must normally be replenished from time to time and it may fairly be assumed that a rise in the serum-inorganic phosphorus is instrumental in this effect leading to a deposition of calcium phosphate in the bones. Regarded from this aspect, the mechanism of the reciprocal relationship might be stated thus: A rise in the serum-inorganic phosphorus leads to a fall in the serum-calcium by deposition of calcium phosphate in bone, while a fall in the phosphorus value, as by excess of excretion over intake, allows liberation of calcium phosphate from the bones to proceed with consequent rise in the serum-calcium value.

Summary.

1. With a view to confirming earlier observations of the effects of the inorganic constituents of diet on the relationships between the concentrations of calcium, magnesium and inorganic phosphorus in the serum of the rabbit, solutions containing the appropriate ions have been injected subcutaneously and their effects on the three constituents compared with those noted previously.

2. The inverse relationship existing between the concentrations of calcium and magnesium in the serum, first observed when rabbits are kept on certain diets, has been confirmed by the subcutaneous injection of calcium and magnesium salts.

3. The inverse relationship between the serum-calcium and inorganic phosphorus and the direct relationship between the serum-magnesium and inorganic phosphorus is only supported in so far as the injection of disodium phosphate

causes a fall in the serum-calcium. The injection of the gluconate, lævulate and lactate of calcium has no constant effect on the serum-inorganic phosphorus, nor does the injection of phosphate affect the serum-magnesium. The injection of the sulphate and lactate of magnesium depresses the serum-inorganic phosphorus, and the relationship is therefore the converse of that observed in the dietary experiments.

4. The above findings are discussed in relation to the concentration of calcium, magnesium and inorganic phosphorus, normally existing in the blood.

5. The injection of sodium sulphate depresses the serum-calcium and inorganic phosphorus but leaves the serum-magnesium unchanged.

6. The injection of sodium chloride has no significant effect on the serum-calcium, magnesium or inorganic phosphorus.

7. Small haemorrhages cause a slight temporary depression of the serum-calcium.

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Je donnerai comme argument une des observations qui ont servi de base à nos recherches communes. Un jeune homme est atteint depuis son enfance de crises d'asthme; son état général s'altère; il tousse et crache; l'examen clinique décèle des signes de catarrhe sibilant diffus; une radiographie donne une image de tramite généralisée, dans laquelle se détache, sous la clavicule gauche, un territoire de périlobulite typique, située à l'extrémité d'un rayonnement trabéculaire fin, partant du hile; les crachats ne contiennent pas de bacilles de Koch, mais l'inoculation, après filtration, donne une tuberculose expérimentale discrète du type de celle qui est déterminée par le virus filtrant. Peu de jours après notre premier examen, il fait une grande hémoptysie fébrile, qui annonce une poussée évolutive; un foyer congestif est constaté sous la clavicule gauche; les crachats contiennent d'abondants bacilles de Koch. Un an après cette poussée, dont l'acuité a été de courte durée, la radiographie montre, au siège de l'ancien territoire de périlobulite sous-claviculaire gauche, une traînée d'opacités confluentes échelonnées le long de grosses travées rayonnantes partant du hile. En somme, au cours de la première phase, le virus filtrant seul existait et la lésion était une zone de périlobulite; ultérieurement le bacille de Koch apparait sous sa forme normale et la périlobulite discrète fait place progressivement à l'infiltration de tout le même territoire.

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RECHERCHES EXPÉRIMENTALES

SUR LES CHOLAGOGUES

ADMINISTRÉS PAR VOIE VEINEUSE

PAR MM.

Etienne CHABROL et Maurice MAXIMIN.

C'est au cours de recherches expérimentales sur la physio-pathologie des sels biliaires que nous avons repris l'étude des cholagogues.

Ce sujet a inspiré de multiples publications souvent contradictoires. Peut-être faut-il en chercher la raison dans la diversité même des techniques qui ont permis de l'aborder. Certains observateurs, à l'exemple de Steinmetzer, opèrent sur des chiens porteurs d'une fistule duodénale. Cette technique, qui offre le précieux

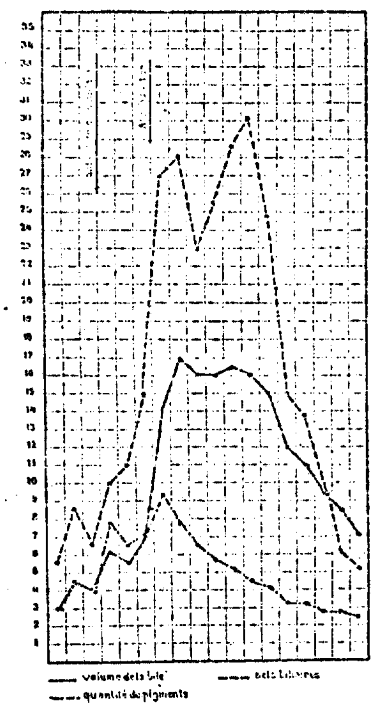

Fig. 1. — Action de l'atophan injecté par voie veineuse, à la dose de 1 gr., chez un chien de 14 kilogr.

avantage de pouvoir conserver longtemps l'animal, a, par contre, le grave inconvénient de laisser échapper une fraction de la bile dans l'intestin. La même critique s'adresse aux expériences sur les animaux porteurs d'une fistule vésiculaire sans ligature préalable du canal cholédoque.

Désireux de recueillir la totalité de la bile, nous n'avions le choix qu'entre deux méthodes : la fistule vésiculaire après ligature du canal cholédoque, et la *fistule cholédocienne après exclusion de la vésicule*.

C'est ce dernier procédé que nous avons adopté, en renouvelant chacune de nos expériences sur un chien neuf et vigoureux; nous avons préféré répéter chaque fois le même geste opératoire, plutôt que d'être exposés aux inconvénients multiples d'une fistule permanente, chez un animal dont la plaie suppure et dont la cachexie ne cesse de progresser. Dans toutes nos expériences, notre idée dominante a été d'obtenir des résultats strictement comparables. C'est dire que nos chiens ont reçu, proportionnellement à leur poids, la même dose d'anesthésique, qu'ils ont été opérés dans les mêmes conditions techniques[1] et que la voie d'administration du cholagogue a toujours été la même. *C'est la voie veineuse que nous avons choisie*; les résultats quasi immédiats qu'elle permet d'obtenir se prêtent à des appréciations qui seraient fort délicates s'il fallait attendre plusieurs heures qu'un cholagogue administré par la bouche fût absorbé par l'intestin.

***

Nous classerons les cholagogues que nous avons éprouvés en prenant comme base leur efficacité.

Différents corps de la série aromatique viennent en tête et, parmi eux, la *phénylquinoline*, l'atophan, comme l'ont montré Brugsch et Horsters. Les investigations de ces auteurs ayant porté sur le volume total de la bile recueillie pendant un laps de temps de six heures, il nous a paru intéressant de suivre de plus près l'évolution des phénomènes. Il est facile d'administrer l'atophan par voie veineuse, sans le moindre incident, sur le chien, à condition de le solubiliser au préalable grâce à l'adjonction de bicarbonate de soude.

Les résultats sont constants. Sur 9 animaux différents, ayant reçu une dose moyenne de 0 gr. 05 d'atophan par kilogramme de poids, nous avons toujours vu augmenter le volume de la bile dans des proportions impressionnantes. Ce volume peut quintupler après la piqûre. L'hypersécrétion est immédiate; quelquefois même, elle atteint son apogée dans la première demi-heure qui suit l'injection; mais, généralement, c'est au bout d'une heure ou deux que l'on note le maximum de l'excrétion biliaire. Celle-ci se poursuit pendant quatre ou cinq heures consécutives, cinq fois plus forte que durant la période témoin. La décroissance se produit ensuite brusquement, le volume de la bile retombant à son chiffre normal en l'espace d'une heure sinon d'une demi-heure (fig. 1).

La cholerragie déterminée par l'atophan n'est pas sans avoir un retentissement sur l'excrétion de la bilirubine, des sels biliaires et de la cholestérine. A l'apogée de la sécrétion, chaque échantillon de bile apparait beaucoup plus clair que les échantillons témoins recueillis de demi-heure en demi-heure avant l'expérience; toutefois, si l'on tient compte du volume total de la bile excrétée, on est conduit à reconnaître qu'il se produit une élimination quasi normale de la bilirubine. Nos courbes sont très précises à cet égard.

L'excrétion des sels biliaires est plus importante, tout au moins au début. Cependant, au fur et à mesure que l'expérience se prolonge, les échantillons recueillis dénotent une tension superficielle de plus en plus élevée qui traduit leur appauvrissement en taurocholates de soude.

L'excrétion de la cholestérine obéit aux mêmes lois. De même que les sels, cette substance ten

1. Nos animaux étaient anesthésiés à la chloralose injectée dans les veines à la dose de dix centigr. par kilogr. de poids. Après ligature du canal cystique, une canule en verre était introduite dans le cholédoque et la bile recueillie de demi-heure en demi-heure dans des tubes gradués. Le dosage des pigments a été effectué par la méthode colorimétrique, en comparant entre eux les différents échantillons de bile et en tenant compte du volume de la bile excrétée.

La stalagmométrie, au moyen du compte-gouttes de Duclaux, nous a donné un aperçu de la teneur de cette bile en sels biliaires. Pour ... les substances ... qui peuvent ... superficielle aux côtés des taurocholates, nous avons toujours eu la précaution de pratiquer nos ... sur ... bile diluée au 1/30 dans de l'eau ... pour le détail de cette technique, voir l'article de MM. Chabrol, Benard et Grandjean, *La Presse Médicale*, 7 juillet 1928.

Il importe de retenir dans ces expériences que la chloralose ... *par elle-même une action cholagogue*. Nous avons prévenu cette cause d'erreur en ne recueillant nos premiers échantillons de bile que deux heures après l'injection de l'anesthésique.

Sult

à tarir en fin d'expérience, chez ces animaux qui ont subi une véritable saignée biliaire. Et nous pouvons trouver dans ces faits une nouvelle démonstration du cycle entéro-hépatique que Schiff a bien mis en lumière.

Nous avons exposé, avec quelques détails, l'action de l'atophan sur la sécrétion hépatique de la bile, cette substance devant nous servir de terme de comparaison dans nos expériences ultérieures.

Elle n'est point la seule, d'ailleurs, qui soit dotée d'un pouvoir cholagogue énergique. Nous avons constaté, avec MM. Charonnat et Porin, que cette propriété appartenait également à d'autres substances de la série aromatique jusqu'à ce jour méconnues : le *naphtoate de soude* et l'*oxy-naphtoate de soude* par exemple. Nous reprendrons ces faits dans un mémoire d'ensemble, où nous rechercherons avec nos collaborateurs si, à côté de l'élément carboxyl, COOH, la grosseur de la molécule n'intervient pas, entre

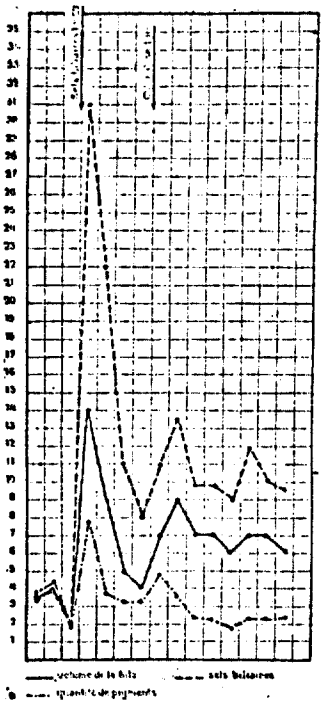

Fig. 2. — Action cholérétique des sels biliaires injectés par voie veineuse à la dose de 1 gr. 50 chez un chien de 20 Kilogr.

autres facteurs, pour expliquer l'action cholagogue vraiment très remarquable de certains corps de la série aromatique.

Les *sels biliaires*, que nous avons injectés à la dose moyenne de 0 gr. 075 par kilogramme de poids à six animaux, nous semblent occuper le deuxième rang par rapport aux substances précédentes. En les administrant par voie buccale, Whipple, Foster et Hooper avaient noté que leur action se manifestait quatre à cinq heures après l'ingestion. Par voie veineuse, c'est immédiatement après la piqûre que nous voyons augmenter le volume de la bile. Celui-ci double ou triple, suivant la dose injectée. Dans son ensemble, la courbe affecte la même allure que celle obtenue sous l'action de l'atophan. Dans les deux cas, la terminaison est très brusque, mais, sous l'effet des sels biliaires, l'ascension est plus faible et la durée plus courte; il n'est pas rare cependant de noter une légère reprise après un temps d'arrêt d'une demi-heure (fig. 2).

Comme on pouvait s'y attendre, la tension superficielle de la bile est remarquablement basse; le très brusque crochet que l'on note sur nos courbes paraît bien correspondre à l'excrétion hépatique de la substance que l'on a introduite dans l'organisme par voie intraveineuse.

On pouvait se demander si l'effet cholagogue des sels biliaires était subordonné à leur pouvoir hémolytique. Il ne le semble pas. Comme le

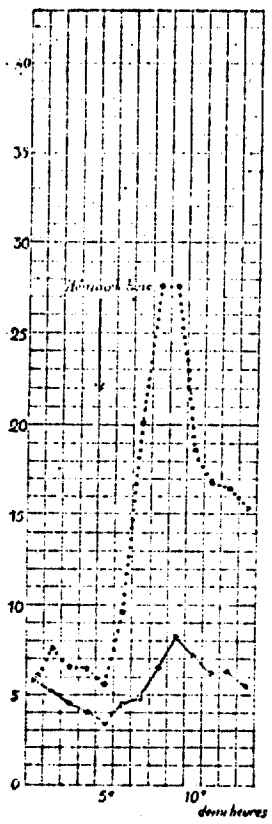

Fig. 3. — Injection intraveineuse d'hémoglobine à un chien de 19 kilogr. (Gilbert, Chabrol et Bénard).

—— Volume de la bile excrétée par demi-heure.
- - - - Quantité de bilirubine excrétée.

montrent nos tracés, l'excrétion des pigments resta sensiblement la même avant et après l'injection durant les premières demi-heures. Lorsqu'il se produisit une hémoglobinémie, son retentissement sur la biligénie pigmentaire fut beaucoup plus tardif. Et, à cet égard, il n'est pas sans intérêt de rapprocher nos observations des résultats expérimentaux que nous avons publiés en 1912 avec MM. Gilbert et Henri Bénard. En injectant à des chiens, par kilogramme d'animal, 12 cmc 1/2 d'une solution d'hémoglobine à 2 pour 100, nous avions vu, une heure et demie après la piqûre, la quantité des pigments quadrupler ou quintupler, alors que le volume de la bile restait sensiblement le même. Nous sommes loin d'observer un résultat comparable avec les sels biliaires; c'est le phénomène inverse que nous notons ici : le volume de la bile peut tripler après une injection de sels, mais l'excrétion pigmentaire reste sensiblement la même qu'avant l'expérience. Si les sels biliaires ont un retentissement sur la biligénie pigmentaire en déterminant de l'hyperhémolyse, ce n'est pas immédiatement, dans les premières heures, que l'on doit en chercher le reflet, c'est beaucoup plus tard et l'on conçoit que cette action hémolytique dépende en très grande part de la dose injectée (fig. 3).

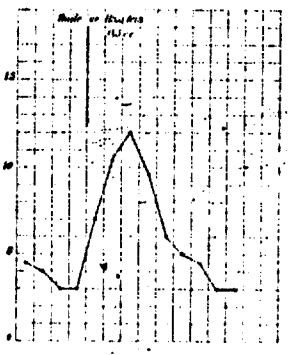

Fig. 5. — Action cholérétique de l'huile de Haarlem.

Ajoutons que sur l'homme comme sur le chien on peut noter les mêmes effets cholagogues à la

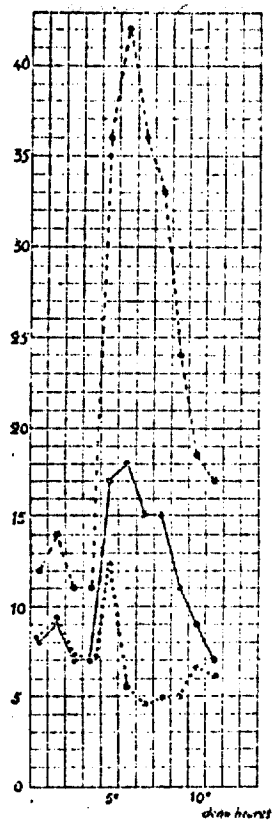

Fig. 4. — Brusque excrétion de bile et de sels biliaires, à la suite d'une injection intraveineuse de 2 gr. de sels, chez une malade soumise à un drainage du cholédoque.

—— Volume de la bile excrétée.
- - - - Quantité de sels biliaires.

suite d'une injection intraveineuse de sels biliaires. Notre courbe n° 4 est celle d'une malade de M. Bergeret qui, au cours d'une angiocholite lithiasique, était soumise à un drainage en T du canal cholédoque. Une injection intraveineuse de sels biliaires tripla le volume de la bile et produisit un fort abaissement de la tension superficielle, sans modifier l'excrétion des pigments (fig. 4).

L'*huile de Haarlem* nous a donné des résultats sensiblement comparables à ceux des sels biliaires, sur deux animaux observés avec M. Porin. L'injection intraveineuse de 1 cmc d'huile de Haarlem à deux chiens du poids respectif de 15 et 19 kilogr. a doublé et presque triplé le volume de la bile excrétée. Les encore, l'excrétion fut immédiate dès la demi-heure qui suivit la piqûre : l'augmentation de la sécrétion biliaire se manifesta durant trois à quatre demi-heures consécutives, la courbe retombant ensuite très nettement à ses chiffres témoins (fig. 5).

Ces constatations furent pour nous le prétexte d'une série de recherches sur l'action cholagogue de différentes huiles et de leurs constituants.

Partant de la notion classique que l'huile d'olive subissait dans l'intestin le phénomène de la saponification, pour donner tour à tour de la glycérine, de l'acide oléique et des oléates, nous avons étudié l'action cholagogue de ces différents éléments en les introduisant dans l'organisme par voie intraveineuse.

L'*huile d'olive*, injectée directement dans le sang à la dose de 2 cmc chez un chien de 16 kilogr., ne nous a fourni aucun résultat durant les deux heures qui ont suivi la piqûre. Le volume de la bile excrétée resta le même que celui des échantillons témoins et, fait intéressant, il ne se produisit aucune modification de la tension superficielle.

La *glycérine*, préconisée jadis par Ferrand comme cholagogue, est restée également sans effets sur deux chiens de 4 et 18 kilogr. qui en avaient reçu respectivement 2 gr. et 5 gr. dans les veines.

Seuls l'acide oléique et l'oléate de soude nous ont paru augmenter la sécrétion hépatique de la bile.

L'action de l'*acide oléique* est tardive. C'est seulement durant la troisième demi-heure que cette substance introduite dans les veines, à la dose de 2 gr. chez un chien de 19 kilogr., fait passer le volume de la bile de 4 cmc à 6 cmc. Celui-ci s'élève à 9 cmc pendant les trente

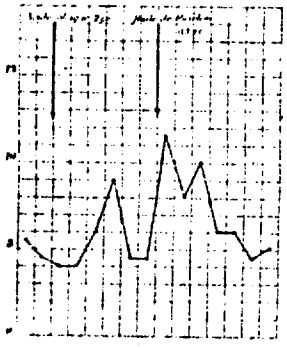

Fig. 6. — Action cholérétique de l'acide oléique et de l'huile de Haarlem.

minutes suivantes et retombe presque aussitôt à son chiffre normal (fig. 6).

L'*oléate de soude* a une action plus immédiate. Celle-ci nous a paru constante, quelle que fût la dose injectée : 20, 40, 80 centigr. d'oléate de soude, chez trois chiens pesant respectivement 22, 21 et 17 kilogr., ont eu l'effet commun de doubler le volume de la bile, comme en témoigne la courbe ci-jointe. Ce résultat constant est cependant minime, lorsqu'on le compare à celui de l'atophan qui est susceptible de quintupler la sécrétion hépatique. Il va de soi qu'au cours de ces expériences sur l'oléate de soude, nous ne pouvions retenir les variations de la tension superficielle pour étudier l'excrétion des sels biliaires.

Relatons enfin, à titre documentaire, que l'*essence de térébenthine*, l'un des constituants de l'huile de Haarlem, n'a paru présenter aucune action cholagogue sur un chien de 16 kilogr. qui avait reçu dans les veines 1/2 cmc de pinène pure.

***

Nous venons de voir que l'atophan, l'oxynaphtoate de soude, les sels biliaires et l'huile de Haarlem étaient au premier rang des cholagogues administrés par voie veineuse : l'atophan quintuple la sécrétion hépatique de la bile, alors que

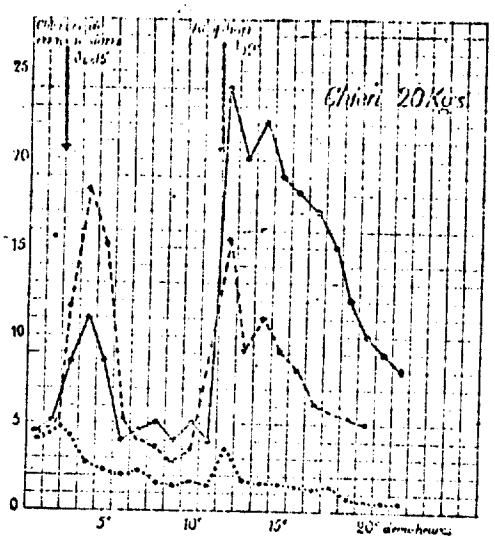

Fig. 7. — Action cholérétique comparée du chlorure de magnésium et de l'atophan.

—— Volume de la bile excrétée.
- - - - Quantité de sels biliaires excrétés.
—·—·— Quantité de pigments.

les sels biliaires et l'huile de Haarlem sont susceptibles de la tripler.

Au second plan, apparaissent une série de substances dont l'action est plus modeste, puisqu'elles se bornent à doubler le volume de la bile (fig. 7).

Nous avons déjà relaté les effets de la *chloralose*, en indiquant notre technique expérimentale, et ceux de l'*oléate de soude*, en comparant cette substance à l'huile de Haarlem. Rapprochons dans le même chapitre le *chlorure de magnésium*,

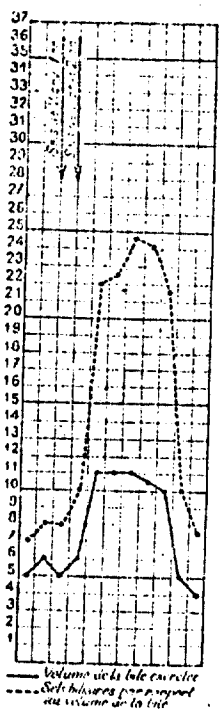

Fig. 8. — Action cholérétique des sels de Vichy injectés par voie veineuse à un chien de 16 kilogr.

le *sulfate de soude* et les *sels de Vichy*. Il serait fastidieux de détailler à leur propos toutes nos expériences. On lira sur nos courbes dans quel ordre de grandeur varient le volume de la bile et la tension superficielle sous l'action de ces trois derniers cholagogues. Notons surtout qu'ils peuvent entraîner des chutes considérables de la tension superficielle (fig. 8).

Quelles en sont les raisons ? A première vue, le sulfate de soude, le chlorure de magnésium et les sels de Vichy n'apparaissent guère comme des dénivelants, à la dose moyenne de 40 centigr. par litre de sang que nous les injectons. Leurs solutions aqueuses à 1 gr. pour 1.000 donnent avec le compte-gouttes de Duclaux les mêmes chiffres que l'eau distillée. Ce ne sont pas davantage des dénivelants indirects de la tension superficielle de la bile. Lorsqu'on les mélange à cette dernière, en proportions variables, on obtient avec le compte-gouttes les mêmes résultats qu'avec les dilutions d'eau moins, préparées en partant de la bile et de l'eau distillée.

Ces données expérimentales nous laissaient supposer que le sulfate de soude, le chlorure de magnésie et les sels de Vichy abaissent surtout la tension superficielle de la bile parce qu'ils provoquent une abondante excrétion de sels biliaires.

Ajoutons que, pour ces différentes substances, il existe une *dose optima* permettant d'obtenir les effets cholagogues. C'est ainsi que le chlorure de magnésium injecté à la dose de 0 gr. 07 par kilogramme ne modifie en rien l'excrétion hépatique de la bile ; nos expériences sont, à cet égard, en complet désaccord avec celles de Laborde, qui prétendait jadis avoir obtenu une hypersécrétion biliaire

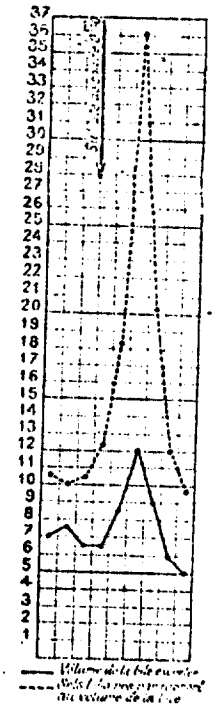

Fig. 9. — Action cholérétique du sulfate de soude injecté par voie veineuse à un chien de 15 kilogr.

importante en injectant à des chiens par voie veineuse 1 et 2 gr. de chlorure de magnésium. C'est seulement à la dose minime de 1 centigr. en moyenne par kilogramme de poids que nous avons vu le chlorure de magnésium manifester une action cholagogue incontestable, confirmant ainsi une expérience antérieure de MM. Carnot et Gachlinger sur un chien porteur d'une fistule duodénale (fig. 9).

C'est peut-être aussi pour une raison de dose optima que le *salicylate de soude* et l'*aspirine* n'ont pas donné, chez nos animaux, le effets cholagogues que nous pouvions attendre

Le salicylate de soude passe pour un cholagogue très actif, s'il faut en croire les expériences déjà anciennes de Levaschew et de Rutherford. Nous l'avons injecté deux fois par voie intraveineuse; il nous a tout au plus permis de doubler le volume de la bile excrétée. Un premier chien du poids de 14 kilogr. reçut 1 gr. de salicylate de soude : le volume de la bile varia de 4 cmc à 8 cmc, de 5 cmc à 7 cmc.

La même substance, administrée à la dose de 1 gr. chez un autre animal du poids de 22 kilogr., donna des résultats très comparables.

L'acide acétylsalicylique est, lui aussi, considéré comme un cholagogue énergique depuis les recherches de Steinmetzer. Or, par voie intraveineuse, à la dose de 1 gr., chez un chien de 22 kilogr., l'aspirine ne nous a donné que de faibles variations du volume de la bile.

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Parmi les substances qui se sont montrées complètement inactives, nous devons mentionner :

L'*acétylcholine*, administrée par voie sous-cutanée à la dose de 0 gr. 05 chez un chien de 21 kilogr.

La *choline* injectée par voie intraveineuse à la dose de 0 gr. 10 chez un chien de 20 kilogr.

La *sécropitnitrine* à la dose de 1 centigr. 1/4 par voie veineuse, chez une chienne de 14 kilogr. 500 en état de gestation. Ce médicament détermina un avortement immédiat, mais ne produisit aucune modification de volume de la bile excrétée. Echec également avec le *bicarbonate de soude* à la dose de 5 gr. par voie intraveineuse chez un chien de 19 kilogr.

Echecs constants, quelle que fût la dose employée, avec le sulfate de magnésie. Nous avons injecté cette substance à 5 animaux qui ont reçu respectivement dans les veines 0 gr. 30, 0 gr. 15 et 0 gr. 01 par kilogramme de poids. Or, jamais nous n'avons obtenu d'hypersécrétion de la bile. Bien plus, il semble même qu'administré par voie veineuse à fortes doses, le sulfate de magnésie ait pour effet de tarir la sécrétion biliaire (fig. 10).

Voici quelques chiffres : le volume de la bile qui oscillait entre 7 et 8 cmc par demi-heure, chiffres témoins, tombe brusquement après l'injection pour se maintenir à 3 ou 2 cmc pendant deux heures consécutives. Cependant cette bile raréfiée est plus foncée qu'avant l'expérience; sa teneur en pigments par centimètre cube passe du simple au triple et sa coloration brunâtre lui donne les caractères d'une bile B dont on ne peut discuter un seul instant l'origine vésiculaire, puisque deux heures auparavant la vésicule a été fonctionnellement supprimée, grâce à une ligature du canal cystique : c'est bien une bile B d'origine hépatique.

Le pouvoir inhibiteur du sulfate de magnésie vis-à-vis de la sécrétion biliaire normale se manifeste encore chez le chien qui a été préalablement soumis à l'action de deux cholérétiques éprouvés, nous voulons parler des sels biliaires et de l'atophan injectés également par voie intraveineuse, à la dose moyenne de 0 gr. 40 par kilogramme.

On verra sur notre courbe n° 10 que l'action cholagogue des sels biliaires est complètement entravée chez le chien qui a reçu quatre heures

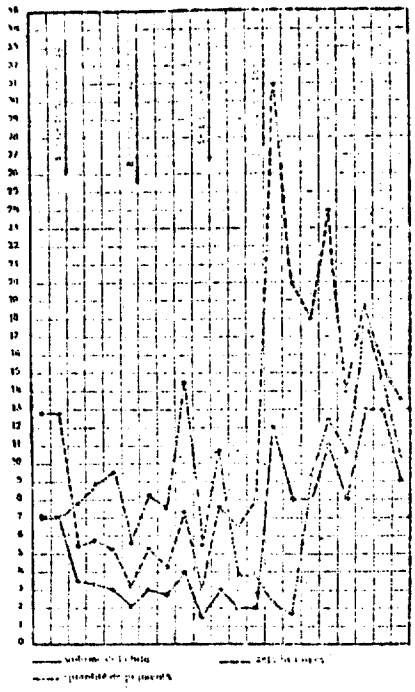

Fig. 10. — Action combinée du sulfate de magnésie, des sels biliaires et de l'atophan chez un chien de 19 kilogr.

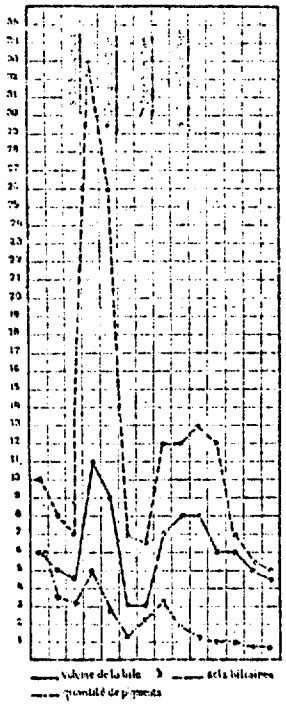

Fig. 11. — Action combinée des sels biliaires, du sulfate de magnésie et de l'atophan chez un chien de 13 kilogr.

auparavant du sulfate de magnésie par voie intraveineuse. C'est seulement après un laps de temps de cinq heures et demie que l'on peut obtenir sur cet animal un effet cholérétique, en lui administrant 1 gr. d'atophan. Encore faut-il noter que sur notre courbe n° 11 cette excrétion est minime, aussi bien en ce qui concerne le volume de la bile que le taux des pigments et des taurocholates, lorsqu'on compare les résultats à ceux qui figurent sur nos courbes témoins (fig. 11).

Nous avons reconnu la même action inhibitrice en injectant le sulfate de magnésie non plus avant mais après les deux cholérétiques. Dans les deux cas, que l'on ait affaire à l'atophan ou aux sels biliaires, la courbe du volume de la bile excrétée tombe immédiatement sous l'effet du sulfate de magnésie.

Ces recherches expérimentales sur le sulfate de magnésie administré par voie intraveineuse nous paraissent offrir un double intérêt :

Elles touchent indirectement à la physio-pathologie de l'épreuve de Meltzer-Lyon, en nous montrant qu'un facteur hépatique est susceptible d'intervenir au cours de cette épreuve. Certes, nous ne saurions assimiler nos injections intraveineuses de sulfate de magnésie chez le chien aux résultats enregistrés chez l'homme à la suite d'une instillation duodénale. Nous pensons même très volontiers, en nous basant sur nos observations cliniques, qu'il est possible d'obtenir par la sonde d'Einhorn une bile B vésiculaire, dont l'origine ne peut être contestée lorsque cette bile B est encadrée par deux témoins A et C beaucoup plus pâles. L'action inhibitrice que nous signalons aujourd'hui sur la cellule du foie n'est pas en désaccord avec l'hypothèse de Vincent-Lyon, lorsque cet auteur prétend que le sulfate de magnésie à pour effet de relâcher le sphincter d'Oddi et de permettre ainsi une brusque évacuation des voies biliaires. Il ne ressort pas moins de nos faits expérimentaux que le sulfate de magnésie, injecté à certaines doses dans la circulation sanguine, modifie sans conteste la sécrétion hépatique de la bile. Einhorn croyait que ce médicament déterminait une hypersécrétion hépatique. C'est la constatation inverse que nous avons notée. Dans les conditions expérimentales où nous nous sommes placés, le sulfate de magnésie nous a semblé diminuer plutôt le volume de la bile sécrétée.

Un deuxième enseignement découle de nos recherches. Elles contribuent à situer le sulfate de magnésie en regard des cholagogues éprouvés que sont l'atophan, les sels biliaires et l'huile de Haarlem, et à opposer suivant la conception très judicieuse de Brugsch les substances cholérétiques, agissant sur le foie, aux cholagogues dont l'effet excrétoire semble se cantonner dans le domaine des voies biliaires extrahépatiques. C'est à ce dernier groupe que semble appartenir le sulfate de magnésie qui réalise ce paradoxe de déterminer un brusque afflux de bile dans l'intestin, tout en ayant à certaines doses un pouvoir inhibiteur sur la cellule du foie.

Nous mentionnons incidemment ces conclusions relatives au sulfate de magnésie, le but de notre mémoire étant surtout de graduer des faits expérimentaux, en se plaçant dans des conditions de technique aussi précises que possible.

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RATE OF EXCRETION OF RADIOACTIVE SULFUR AND ITS CONCENTRATION IN SOME TISSUES OF THE RAT AFTER INTRAPERITONEAL ADMINISTRATION OF LABELED SODIUM SULFATE

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A study of the distribution of S^{35} in the rat, 14 to 16 hours after the intraperitoneal administration of sodium sulfate, labeled with S^{35}, has been reported by Singher and Marinelli (1). Of particular interest is their observation that the highest concentration of the radioactive sulfur was found in the bone marrow. This observation stimulated us to determine the change with time in the concentration of S^{35}, given as labeled sodium sulfate, in the bone marrow of the rat and to relate this changing concentration with the concentration in other tissues, particularly the blood.

Only a brief report (2) was found in the literature on the excretion in urine of S^{35} ingested as sodium sulfate. The rate of excretion of S^{35} in the urine and feces of rats after intraperitoneal injection of labeled sodium sulfate was, therefore, also determined.

EXPERIMENTAL

Adult rats, 180 to 330 gm. in weight, from the colony of Professor E. V. McCollum, were each given 1 mg. of sodium sulfate (in 1 ml. of distilled water), labeled with radioactive sulfur (S^{35}),[1] by intraperitoneal injection. They were then placed in individual metabolism cages, with food and water, to allow for the separate collection of urine and feces. The cages had been so designed by Professor McCollum and his coworkers that there was a minimum of food spillage, and therefore contamination, of urine and feces with food. The rats were sacrificed at intervals of time, as follows: A rat was anesthetized in an ether jar. The heart was exposed and as much blood as possible was withdrawn directly from the heart

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[1] The S^{35} used in this investigation was supplied by the Clinton Laboratories, Monsanto Chemical Company, and obtained on allocation from the United States Atomic Energy Commission.

Sulf.

with a hypodermic needle and syringe. A portion of the liver and the brain were then removed and placed in small vials. Weighed samples of these tissues, the feces collected in the time the rat sojourned in the metabolism cage, and 1 ml. of the blood were placed in separate nickel crucibles, each of which contained 2 ml. of 10 per cent sodium hydroxide and 4 gm. of anhydrous sodium carbonate. The crucibles were placed in a drying oven at 110–120° until dry. The dry material in each crucible was oxidized with sodium peroxide according to Bailey (3).

The humeri, femurs, and tibiae were freed of muscle and periosteum. The epiphysis of each bone was cut off at its junction with the diaphysis. The diaphysis of each bone was then freed of its marrow by pushing the latter out with a stainless steel wire. This marrow was placed immediately in a small screw cap vial and weighed. The residual marrow in the bone was wiped out by pushing through a small plug of moist cotton repeatedly until no further marrow was in evidence. As a check, each bone was split and carefully examined. It was further cleaned, if necessary, by wiping with moist cotton. The bone shafts were combined and powdered in a stainless steel mortar with a stainless steel pestle. A weighed portion of the powdered bone was oxidized with Benedict-Denis reagent (4).

The weighed bone marrow was extracted 3 times with at least 10 times its weight of 5 per cent trichloroacetic acid in a centrifuge tube. The combined extracts and the residue were each neutralized with 10 per cent sodium hydroxide, with phenolphthalein as an indicator, and then oxidized with Benedict-Denis reagent (4).

The urine excreted by each rat was combined with the washings of the metabolism cage and diluted to a volume of 100 ml. The total sulfur in aliquots of the urine was oxidized to sulfate according to Denis (4).

Before precipitation of sulfate from any sample as barium sulfate, 5 ml. of 0.05 N sodium sulfate solution were added to each sample so as to bring the final weight of barium sulfate to about 30 mg. Precipitation of barium sulfate was allowed to proceed for 16 to 20 hours at room temperature.

All barium sulfate samples were isolated by centrifugation and, after washing with distilled water in the centrifuge tubes, transferred to counting cups as slurries in 70 per cent ethanol as previously described (5).

The activity of each sample was determined with mica end window (2.8 mg. per sq. cm.) Geiger-Müller tube (Victoreen) and a Cyclotron Specialties scaler. At least two separate determinations of the activity of each sample were made for a sufficiently long period of time to obtain a precision of about 2 per cent. All values were corrected for decay and self-absorption.

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RESULTS AND DISCUSSION

Of the twenty-seven rats used, fourteen were males and thirteen were females. The points given in Figs. 1 to 3 are average values, calculated from results on four animals at 4 hours, three animals at 8 hours, four animals at 16 hours, eight animals at 24 hours, and two animals each at 48, 72, 96, and 120 hours.

Fig. 1 shows the rate at which the S^{35} was found to be excreted in the urine and feces of the rats. Approximately 67 per cent of the activ-

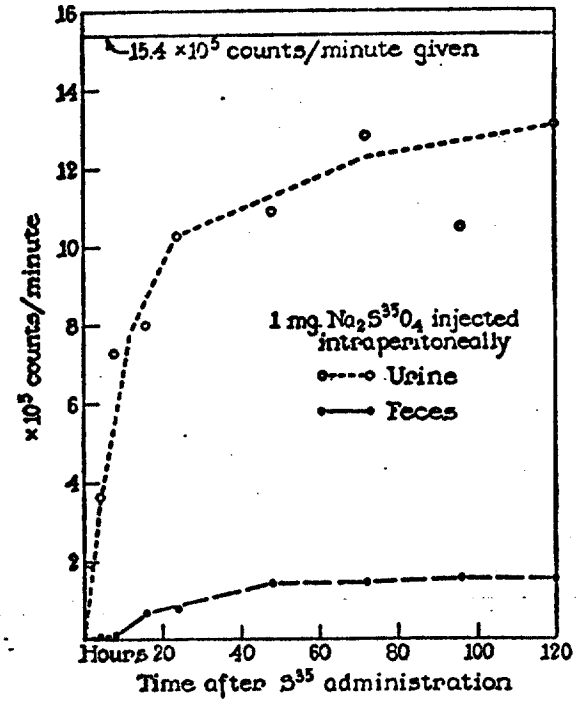

FIG. 1. Excretion of S^{35} in the urine and feces by adult rats after intraperitoneal injection of labeled sodium sulfate.

ity injected was excreted in the urine by the end of the 24th hour. Borsook *et al.* (2) could account for only 47 per cent at the end of 24 hours in the urine of a man. The subject studied by Borsook *et al.* ingested the labeled sodium sulfate by mouth. The rats studied by us received the sodium sulfate containing S^{35} by intraperitoneal injection. This difference in the route of administration may account for the difference in the fraction recovered at the end of 24 hours. On calculating the intake by the human subject and by the rats, assuming that the human subject weighed somewhere within the limits of 45 to 90 kilos, one

arrives at an intake of approximately 9.8 to 19.7 mg. of sodium sulfate per kilo as compared to approximately 4 mg. per kilo by the rats. This difference in intake might also be considered as a possible explanation for the difference in amount of S^{35} found excreted in the urine within 24 hours. One should, however, consider in addition a possible species difference as regards rate of sulfate excretion.

By the end of the 120th hour after the administration of the labeled sodium sulfate, the activity of S^{35} recovered in the urine accounted for approximately 85 per cent of the activity given. Approximately 95

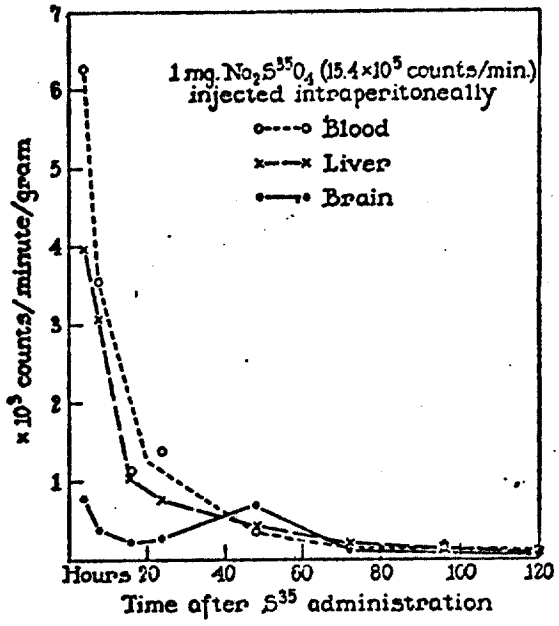

FIG. 2. Retention of S^{35} in the blood, liver, and brain by adult rats after intraperitoneal injection of labeled sodium sulfate.

per cent of the S^{35} is accounted for at the end of 120 hours if the activities found in the urine and feces are added.

In Fig. 2 are presented curves showing the change in concentration of S^{35} with time in the blood, liver, and brain. From these curves it would seem that sulfate sulfur as such, when it enters the circulation or the liver in a normal rat, is rapidly eliminated from these tissues. Whether the brain is similar in this respect or whether it slowly accumulates S^{35} presented to it as sulfate sulfur is difficult to say without extending the number of observations reported here. In any case, the concentration of S^{35} in the liver and blood appeared to have reached a similar concentration at about the 48th hour after the injection of the

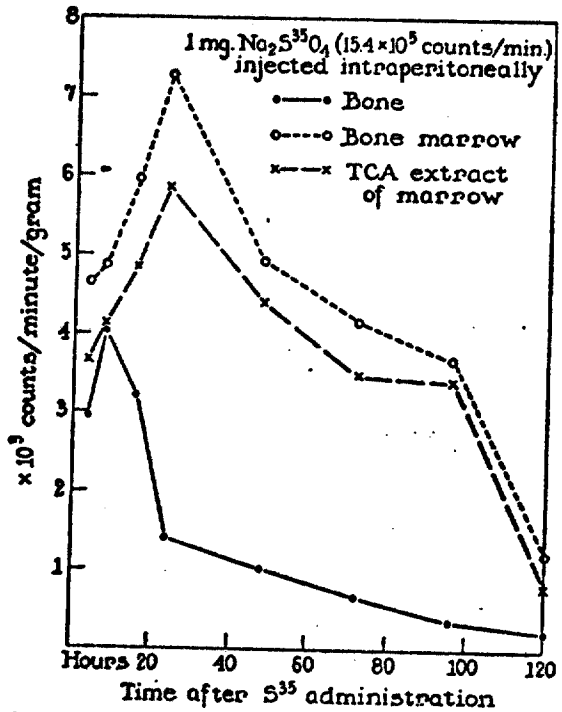

FIG. 3. Retention of S^{35} in the bone and bone marrow by adult rats after intraperitoneal injection of labeled sodium sulfate.

TABLE I

Extent of S^{35} Removal from Rat Bone Marrow by 3-Fold Extraction with 5 Per Cent Solution of Trichloroacetic Acid

A male rat, 355 gm. in weight, was used 24 hours after the intraperitoneal administration of 1 mg. of sodium sulfate, containing S^{35} (15.4 × 10^5 counts per minute). The values for activity are corrected for radioactive decay and self-absorption.

Sample	counts per min. per gm.
1st three trichloroacetic acid extracts combined	4900
4th trichloroacetic acid extract	0–10
Three alcohol-ether (1:3) extracts combined	64
Residue	793

labeled sulfate. A similarity in the concentration of S^{35} in the brain and blood, from the observations thus far made, was more slowly attained; a similar concentration was reached at about the 72nd hour.

In contrast to the rapid fall of the S^{35} concentration in blood and

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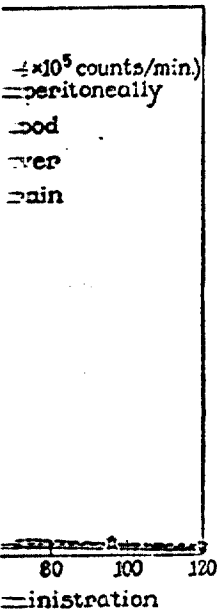

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liver, after intraperitoneal injection of labeled sodium sulfate, is the pronounced rise in the concentration of S^{35} found in bone and bone marrow (Fig. 3). The highest concentration in the bone was observed at about the 8th hour, that in the bone marrow at about the 24th hour after injection of the labeled sodium sulfate. The subsequent drop in the S^{35} concentration in these tissues is also less rapid, particularly in the bone marrow. Even at the end of 120 hours, on a weight basis, the S^{35} concentration is about 2 times as high in the bone and nearly 12 times as high in the bone marrow as in the blood. The observations confirm the report of Singher and Marinelli (1) that the concentration of S^{35}, after administration of labeled sodium sulfate, is higher in bone marrow than that found in most of the other tissues of the rat. The major portion of the activity found in the bone marrow is in a compound or compounds which are soluble in a 5 per cent solution of trichloroacetic acid. That a 3-fold extraction of bone marrow with a 5 per cent solution of trichloroacetic acid, as employed, was effective in removing all or nearly all of the S^{35}-containing material, which was soluble in this solution, is indicated in Table I. Further work on the characterization of the materials containing S^{35} in the various fractions listed in Table I is contemplated.

SUMMARY

The excretion by rats in urine and feces of S^{35} given in the form of sodium sulfate appears to be rapid. Excretion by these routes accounts for the major portion of the S^{35} given. By the end of the 120th hour approximately 95 per cent was found to have been eliminated by these routes.

In the period of 120 hours the concentration of S^{35} in the blood, liver, and brain was found to have fallen to relatively low levels. A similar concentration of S^{35} in the blood and liver was attained by the 48th hour, in the blood and brain by the 72nd hour.

In contrast to the rapid fall observed in the liver and blood, the concentration of the S^{35} was found to increase until about the 8th hour in bone and until about the 24th hour in bone marrow. The subsequent fall in the S^{35} concentration of the bone and bone marrow was also slower than that in blood, liver, and brain.

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SOME FACTORS AFFECTING THE ACIDITY OF URINE IN MAN

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The recent failure to find any satisfactory explanation of the increase in urine acidity resulting from ingestion of alcohol (Eggleton, 1946) suggested the necessity for an investigation into possible different types of urine acidity. In the experiments with alcohol, the usual criterion of urine acidity, namely changes in hydrogen-ion concentration, was adopted. In such a buffered solution as urine, however, it is apparent that an increase in hydrogen-ion concentration need not necessarily be due entirely to an increase in excretion of acidic ions: a reduction in the overall buffering power of the solution would enable an unchanged excretion of acidic ions to produce a shift in hydrogen-ion concentration.

Hitherto, little attention has been paid to changes in the total buffering capacity of the urine under various physiological conditions, interest being centred rather on the 'titratable acidity' value. This value is obtained by titration of the sample with standard alkali to about pH 8 (phenolphthalein first pink) and represents the buffering power of the urine over the range pH 8 to the pH at which it was secreted. It is found, naturally enough, to vary directly with the hydrogen-ion concentration, i.e. the lower the pH of the urine sample, the higher its 'titratable acidity' value. It gives no indication of the total buffering capacity of any sample over the whole physiological range pH 4·8–8·0 except for those samples secreted at pH 4·8.

Approach from a completely different angle, that of determination of changes in output of phosphate, suggests that an increase in hydrogen-ion concentration of the urine *may* in some circumstances be associated with an increase in total buffering power. Thus Haldane (1921) noted an increase in phosphate excretion following ingestion of ammonium chloride in man, a result later confirmed by Loeb, Atchley, Richards, Benedict & Driscoll (1932), while Gamble, Ross & Tisdall (1923) observed a similar result after fasting, the increase in phosphate output being greater than that accounted for by wastage of the muscles. Under both sets of conditions, ammonium chloride acidosis and fasting, the prime need of the body is to rid itself of excess acidic ions: by

simultaneously increasing the buffering power of the urine, this end can be achieved without increasing the hydrogen-ion concentration beyond the physiological limits to which the kidney is accustomed.

The results to be presented are concerned with experiments designed to test the possible existence of two distinct types of urine acidity on the lines suggested above.

METHODS

The general procedure used in previous investigations was adhered to, with only slight modifications. The meal previous to the experiment, either breakfast or lunch, was omitted, whether the experimental substance was to be ingested or injected, and a glass of water was usually taken 2–2½ hr. before the experiment began. At least one urine sample was collected during the last 30–40 min. of this resting period, and five or six samples during the 2½–3 hr. following the experimental injection or ingestion. A large number of experiments were performed on one subject and these were repeated, whenever feasible, on groups of other subjects.

In addition to determinations of pH and ammonia concentration (formol titration method) as detailed elsewhere (Eggleton, 1946), the buffering power of the urine was determined. To the 'titratable acidity' value (urine titrated to pH 8·0) was added what might reasonably be called the 'titratable alkalinity' value (urine titrated to pH 4·8, using methyl red-methylene blue indicator), the sum of the two representing the buffering of the urine over the physiological range pH 4·8–8·0. Neither titration is extremely accurate, owing partly to the yellow pigmentation of the urine. This inaccuracy was partially counteracted, and reasonably large titration values obtained even on dilute urine samples, by the use of volumes varying from 5·0–50 c.c. according to the rate of urine flow, the smaller samples being diluted to 50 c.c. in each case. In a few experiments determinations were also made of phosphate output.

RESULTS

The results obtained by Haldane and his successors in regard to the action of acidifying agents such as ammonium chloride were fully confirmed. Two experiments on one subject and one experiment on a second subject were made with ammonium chloride, and one experiment on each of three subjects with ammonium sulphate. In all six a result similar to that shown in Fig. 1 was obtained. Since these substances can be ingested only in dilute solution without inducing vomiting, and since it was desirable, for a reason to be explained later, that they should be taken with a minimum amount of water, they were ingested in the form of enteric-coated capsules. It is clear that, under such conditions of acidosis, a fall in urine pH is accompanied by a pronounced increase in output of buffering substances, of which phosphate accounts for 50–70%. An attempt was made at a later date to give an intravenous injection of hypertonic ammonium sulphate (20% solution) but proved fruitless: nervous symptoms (incipient black-out) and thrombosis occurred after the first c.c. had been injected.

Under all other conditions in which a change in urine pH was observed, a relationship, the exact converse of that shown in Fig. 1, was seen to exist. In Fig. 2, for example, is shown the effect of an intravenous injection of hypertonic sucrose. As the urine acidity increases, the buffer output falls. This experiment was not attempted on any further subjects since it induced

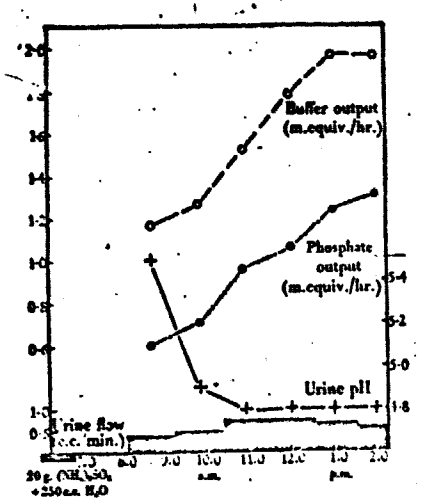

Fig. 1. The effect of ingestion of 20 g. ammonium sulphate (40 enteric-coated capsules) on urine pH, output of phosphate and total buffers.

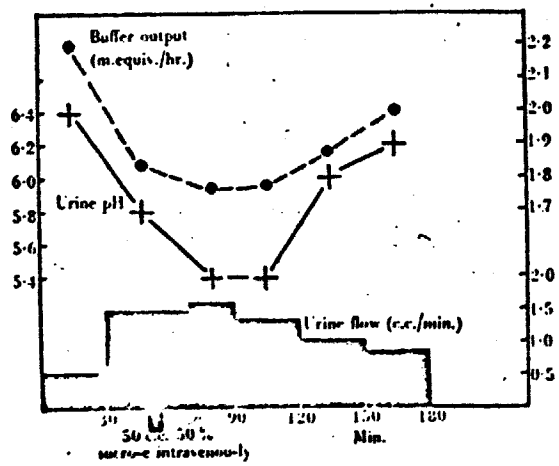

Fig. 2. The effect of intravenous injection of concentrated sucrose solution on urine pH and buffer output.

thrombosis. Hypertonic sodium sulphate, however, was found to produce no ill effects and the average results of an intravenous injection of this substance into four subjects is shown in Fig. 3. Again, the buffer output falls as the urine acidity increases. In a fifth subject the pH fell from 5·6 to 4·8 following the injection, but began to rise again after 30–40 min., and the result, therefore, was not included with those of the remaining four who each individually showed the more prolonged fall seen in Fig. 3. In this figure also is shown the

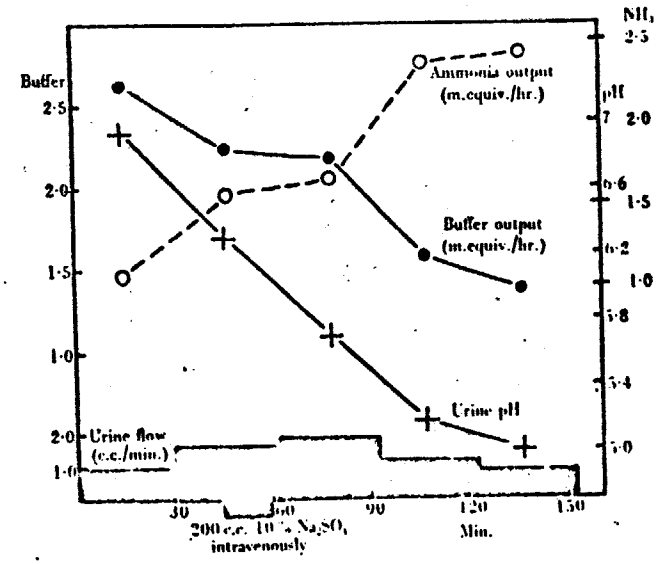

Fig. 3. The effect of intravenous injection of hypertonic sodium sulphate solution on urine pH, buffer output and ammonia output. Average results from four subjects.

inverse relationship which is regularly observed between the outputs of buffer substances and ammonia, a result confirming that previously established between urine pH and ammonia output in alcohol diuresis (Eggleton, 1946).

In contrast with the increase in urine acidity and decrease in buffer output induced by intravenous injection of hypertonic solutions of sucrose or sodium sulphate are the effects, shown in Fig. 4, of the administration by mouth of hypertonic urea solution (the average results of five subjects), but again it is seen that urine pH and buffer output run hand in hand. In one subject an attempt was made to give a rapid intravenous injection of 20% solution of urea; this procedure, however, was not only followed by thrombosis but was so painful that the somewhat erratic course of both urine pH and buffer output which resulted was discounted as being probably connected with the initial nervous disturbance: nor was the experiment repeated on any other subjects.

Ingestion of hypertonic solutions does not necessarily lead to the results shown in Fig. 4. On one occasion, administration of 200 g. glucose in 200 c.c. solution resulted in a fall of urine pH from 6·6 to 4·8, which persisted for the following 2 hr., during most of which time glycosuria was present and the rate of urine flow never greater than 0·63 c.c./min. The experiment was performed with another end in view and buffer output was not determined; the results, therefore, do not merit more than passing mention.

The relationship between buffer output and urine acidity demonstrated in the preceding figures has been observed in some thirty to forty subjects under a variety of conditions, including the naturally occurring variations in acidity

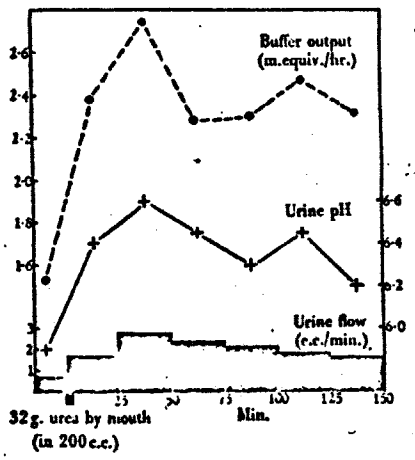

Fig. 4. The effect of hypertonic urea solution taken by mouth on urine pH and buffer output. Average results from five subjects.

encountered during the course of the day. Two factors may, however, mask this relationship at times. Ingestion of tea or coffee results in a disproportionately great output of buffer substances in relation to the pH, but this factor has been eliminated in the series of experiments under consideration. The second factor is that of diuresis itself. A hint of this is to be seen in Figs. 3 and 4 and is shown in pronounced form in Fig. 5. These results again are the average from four subjects who on one occasion took water alone and on another day water, immediately preceded by subcutaneous injection of 1 unit 'pituitrin'. Considerable variations in urine acidity occurred in the different subjects, but in each individual the curve of buffer output followed fairly closely that of urine pH when the rate of urine flow was low. In the water diuresis, however, the large increase in rate of flow, accompanied by a slight fall in urine pH, induced a large increase in buffer output.

This 'flushing-out' effect on buffer substances at high rates of urine flow is sufficiently great to mask the increased acidity following injection of hypertonic sodium sulphate solutions under certain conditions. Two experiments were performed with a different technique from that used on earlier occasions, in an effort to reduce the lag in time between collection of the resting urine sample and injection of the solution; also, the solution was more concentrated (100 c.c. of 20% sodium sulphate) and given in a shorter time in the hope that a more dramatic change in urine acidity might be observed. But these precautions defeated their own ends. In one subject the rate of urine flow rose to

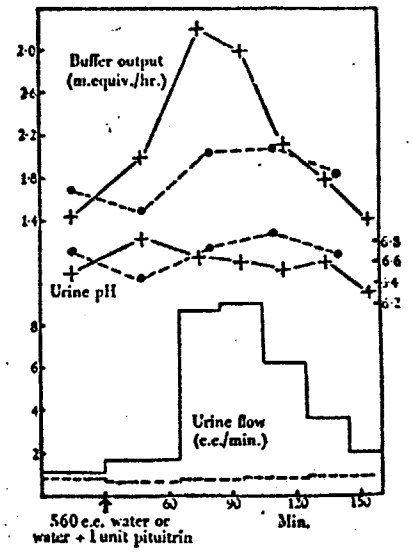

Fig. 5. The effect of rate of urine flow on buffer output. Average results from four subjects +—+ 560 c.c. water; • - - - • 560 c.c. water preceded by 1 unit 'pituitrin' subcutaneously.

7 c.c./min. and in the other to 4·7 c.c./min. following the injection, and in both subjects this relatively large flow was accompanied by an increase in buffer output and no detectable change in urine pH (7·25 and 7·1 respectively in the two subjects). Incidentally, both subjects noted a sensation of thirst before the end of the injection and both developed a severe headache. The latter symptom had also been observed previously in a subject (probably a mild case of diabetes insipidus) in whom both alcohol and water produced a rapid shift in body water, as indicated by an unusually large and speedy diuresis.

Ammonia excretion. Earlier results (Eggleton, 1916) indicated that the output of ammonia is fairly closely correlated with urine pH, the ammonia rising

as the pH falls: and that, although this ammonia output is affected to some extent by varying rate of urine flow, no definite relationship exists between ammonia concentration and urine pH. Those conclusions were drawn from experiments on one individual, and it has been deemed advisable to extend the observations by experiments on a group of other subjects and in reference to urine acidity induced by a number of different agencies.

The results already presented in Fig. 3 show the relationship between ammonia output and urine pH in a group of four subjects after injection of sodium sulphate. Ammonia concentration can readily be calculated from the data given in the figure, and shows no consistent relationship with the pH;

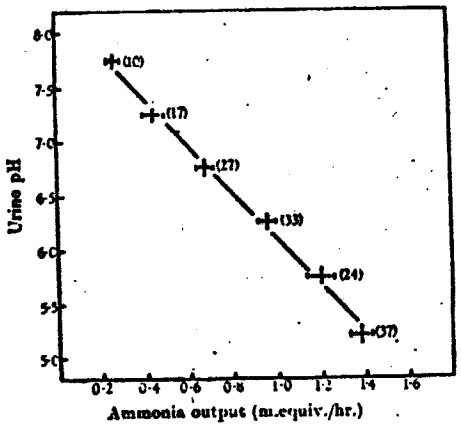

Fig. 6. The relationship between ammonia output and urine pH in one subject, under a variety of conditions. Average values are given for each half unit of pH. The numbers in brackets indicate the number of observations included in each average: the distance between the small vertical lines the standard error of each average. Rate of urine flow varied fifty-fold, from 0·25 to 11·0 c.c./min. in different experiments.

during the stage of increased rate of urine flow, the ammonia concentration falls as the pH falls (from 21 to 15 m.equiv./l.) and during the later stage of diminishing rate of flow, the concentration rises considerably (from 33·5 to 46 m.equiv./l.) as the pH changes only from 5·15 to 5·0.

In thirteen out of thirty subjects on whom at least two experiments have been performed, this relationship between ammonia output and urine pH has been observed. In regard to the remaining seventeen subjects, no such definite conclusion could be drawn. Considerable variation was noted among the whole group of subjects in the extent to which rate of urine flow affected the ammonia output, and only a more intensive study of each subject over a wide range of pH and rate of flow could decide which in each is the more fundamental relationship.

In the subject, for whom data have already been presented from experiments with water and alcohol diuresis (Eggleton, 1946), it has been found that the relation between ammonia output and urine pH remains unaffected when changes in pH are induced by ingestion of urea, or injection of urea, sucrose or sulphate. This relationship, obtained from a large number of experiments of different types, is shown in Fig. 6 in the form of average values for each half unit of pH. The values for ammonia were all obtained by use of the formol titration method, so that the curve for true ammonia would lie about 0·2 m.equiv./hr. to the left of that given.

The results of the experiments in which acidifying agents were ingested have not been included in the averages shown in Fig. 6, for under those conditions the ammonia output is greatly in excess of the values found under other conditions of urine acidity. In the experiment illustrated in Fig. 1, for example, the ammonia output reached a value of over 3 m.equiv./hr.

DISCUSSION

The main point of interest in the results presented is the practical one—that increased urine acidity (hydrogen-ion concentration) may in some circumstances be associated with an increase, and in other circumstances with a decrease in output of buffer substances. The former of these two conditions has been recognized by many workers, but attention has not previously been called to the latter, which appears to be of more general occurrence.

This differentiation between two distinct types of urine acidity argues against Briggs's theory (1934) that the acidifying action of sodium sulphate is due to preferential excretion of the foreign sulphate ion. If that were the case, the increased acidity should be accompanied by an increase in buffer output as with ammonium sulphate, but the reverse is observed.

The fact that sodium sulphate and sucrose, both slowly diffusing substances, lead to an increased acidity of the urine whereas rapidly diffusing urea has no such action suggests that the acidity may be connected with dehydration of the tissues in general. It is tempting to speculate that the post-pituitary anti-diuretic hormone may itself be concerned, though the experiments reported here provide no direct evidence on the point. The relatively small diuresis resulting from injection of sodium sulphate or sucrose (although both should be strong osmotic diuretics) as compared with the larger diuresis after ingestion of urea suggests that secretion of the anti-diuretic hormone has been stimulated by the former. This interpretation is fully in accord with Verney's results (1946) on the unanaesthetized dog; intra-carotid injection of hypertonic urea was found to be without effect on a water diuresis whereas hypertonic sucrose, glucose or NaCl produced immediate inhibition. The result of injections of 'pituitrin' might also be cited as additional evidence in favour of this interpretation. The average results of four subjects, given in Fig. 5, show no large

variations, either in rate of urine flow or in urine acidity, owing to individual variations in the degree of response to water plus 'pituitrin', but in each subject the urine pH followed closely the changes in rate of flow.

If this tentative hypothesis be accepted at the moment for lack of a better one, it should be stressed that the acidifying action of 'pituitrin' cannot override an alkalosis of the body. In two subjects to whom 10 g. potassium citrate had been administered, the resulting diuresis was partially counteracted by subcutaneous injection of 1 unit of 'pituitrin'. Under these conditions the diminished rate of urine flow was accompanied by a decrease in acidity: the pH, which had fallen to 7·5 at the height of diuresis, returned to pH 8·0 after the 'pituitrin' injection.

Any attempt at a theoretical consideration of the results from the *renal* point of view at first seemed impossible, since the excretion of individual buffer substances, and carbon dioxide partial pressures, had not been determined. Some further analysis can, however, be made and I am indebted to Dr L. E. Bayliss for surveying the results and presenting the following interpretation.

The rate of acid or alkali excretion may be readily calculated from the data in this paper if some assumptions are made as to the initial hydrogen-ion concentration of the glomerular fluid, and as to the changes in hydrogen-ion concentration that would result from the re-absorption of water alone. It is reasonable to suppose that the glomerular fluid has a pH of about 7·4, and that the increase in ionic strength, and reduced activity of the water, during elaboration of the urine will not change this by more than 0·1 pH, but this requires further investigation. The amount of buffer substances excreted, as given in this paper, is measured as the amount of acid required to change the pH of the urine from 4·8 to 8·0, i.e. by 3·2 pH units. As a first approximation, therefore, the amount of acid that must have been added by the tubules, or alkali removed, to bring the urine to any observed value of pH is given by

$$(7{\cdot}4 - \text{pH}) \times \frac{(\text{buffer output})}{3{\cdot}2}$$

(it is assumed that the titration curve of urine is a straight line, which is justifiable over the range of pH considered). It may be remarked in parenthesis that, if this viewpoint be adopted, the rate of acid excretion can be directly measured by titrating the urine to pH 7·4, the titre then giving the acid output, and this, divided by (7·4 *minus* pH of urine as excreted) giving the buffer value.

Calculation of the rate of acid excretion by this method leads to the following conclusions:

(1) Administration of ammonium sulphate led to a considerable increase in the acid output (approximately three-fold), as is obvious from the simultaneous rise in buffer output and fall in pH; this increase was maintained after the diuresis had subsided.

(2) Administration of sucrose or sodium sulphate also led to an increase in acid output (by 50–100%); this acid output fell as the diuresis subsided, but less rapidly. The two types of response to administration of sodium sulphate in respect to changes in pH, the one shown in Fig. 3 (four subjects) and the other in a fifth subject mentioned in the text, had the same type of response in respect to acid output; it is the excretion of buffer substances that behaved differently.

(3) Administration of urea led to no appreciable change in the output of acid, the fluctuations in pH being due entirely to fluctuations in buffer output.

(4) During simultaneous administration of water and pituitary extract, there were small and irregular changes in acid output, which were associated with *inverse* changes in the rate of urine flow. This association broke down on the onset of water diuresis, and in one subject in which the urine went alkaline to pH 7·2.

SUMMARY

1. The increased acidity of the urine after ingestion of ammonium sulphate or chloride is accompanied by an increase in buffer output (titration range 4·8–8·0), of which phosphate accounts for about two-thirds (Fig. 1).

2. Intravenous injection of hypertonic sucrose or sodium sulphate solutions also results in increased acidity of the urine, but this is accompanied by a decrease in buffer output (Figs. 2 and 3).

3. The more pronounced diuresis resulting from ingestion of hypertonic urea solution is accompanied by a decrease in urine acidity, with increase in buffer output (Fig. 4).

4. This inverse relationship between urine acidity (hydrogen-ion concentration) and buffer output may be masked at high rates of urine flow by a 'flushing-out' effect on buffer substances (Fig. 5).

5. Evidence is adduced in favour of the tentative hypothesis that increase in urine acidity, accompanied by decrease in buffer output, may be connected with secretion of anti-diuretic hormone by the post-pituitary.

6. A direct relationship is found between urine acidity and ammonia output under all conditions (Fig. 6).

I wish to thank Dr R. A. Gregory and Dr D. R. Wilkie for their generous help, during the earlier and later stages of this research respectively, in giving all the intravenous injections. I am greatly indebted to Dr J. W. Trevan for a supply of enteric-coated capsules prepared at the Wellcome Laboratories.

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The Distribution and Excretion of S^{35} Sodium Sulfate in the Albino Rat[1]

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Introduction

It is generally agreed that inorganic compounds of sulfur, regardless of the route of administration, are rapidly excreted in the urine and feces, with the greatest amount appearing in the urine. This has been demonstrated in the rat by Dziewiatkowski (1) and Tarver and Schmidt (2). Borsook *et al.* (3) and Greengard and Wooley (4) have confirmed this high urinary excretion rate for sulfur in man. Hanahan and Everett (5) showed that in the rat the excretion of sulfate liberated from S^{35} sodium estrone sulfate given subcutaneously and intravenously is likewise rapid and that a large proportion of the administered tagged sulfur appeared in both the urine and feces. In addition, the latter (5) demonstrated that although much of the sulfur entered the intestine through the bile, a considerable amount was excreted in the feces in bile fistula animals. Thus it is apparent that there is a pathway of entry for sulfate into the intestine other than the biliary route.

The present study is concerned with certain detailed aspects of sulfate excretion in rats following intravenous administration and includes efforts to localize routes of sulfur entry into the gastrointestinal tract of bile fistula animals.

Methods

A total of 27 adult female rats were used in this study. These animals were of the Sprague-Dawley strain and ranged in weight from 210 to 230 g. Bile fistulas were prepared as described previously by Hanahan and Everett (5). After recovery, a

[1] This investigation was supported (in part) by a research grant from the National Institutes of Health, U. S. Public Health Service.

ɛtion of S^{35} Sodium Sulfate ɔino Rat[1]

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ɔe 26, 1951

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by a research grant from the National ɛice.

single intravenous injection of S^{35} sodium sulfate[2] was administered. The dosage was metered throughout the experiment to contain 15×10^4 counts/min. which was approximately 1 μg. of sulfate. The animals were kept unrestricted in metabolism cages designed for urine and feces collection and were allowed food and water *ad libitum*.

Animals were sacrificed at intervals ranging from 1 to 24 hr. after the administration of sulfate, and measurements were made for each rat of the amount of S^{35} excreted in the urine, bile, and feces, and of the distribution of tagged sulfur in the gastrointestinal tract, liver, and blood. In addition, the pancreas, parotid glands, and tissue fluids were assayed for S^{35} at certain of the early intervals. Upon sacrificing, a 1-ml. sample of blood was taken by heart puncture and the organs for assay were removed as blood-free as possible. The small intestine was divided into equal thirds and these segments and the other organs were weighed immediately. Aqueous homogenates of the organs and feces were prepared by grinding a 1-g. aliquot in a ground-glass blender. The samples of blood or of organ homogenate were then diluted to 15 ml., and the urine and feces were diluted to obtain desired counting rates. One-ml. aliquots were plated in cupped planchets and dried under an infrared lamp. Control platings to serve as standards and to correct for self-absorption and decay were prepared by adding 1 ml. of a dilution of the S^{35} used for the injection to duplicates of each sample. Similar direct plating methods for S^{35} have been used successfully by Kelsey (6) and Davis (7).

Measurements of radioactivity were made by conventional methods using a 1.7 mg./cm.^{2} mica end window counter.

A comparison of our direct plating procedure with methods of sulfate precipitation by barium [Tarver and Schmidt (2)] or benzidine [*Henriques et al.* (8)] showed the percentage recovery of S^{35} to be comparable. Attempts were made to use protein-free filtrates for counting, but repeated washings failed to extract all the activity.

Results

The data are summarized in Table I and in the graph (Fig. 1). Due to technical difficulties, a complete series of determinations was not always accomplished on the entire series of rats sacrificed at a given time interval. Where this is the case, the number of animals for which satisfactory measurements were obtained is indicated in parentheses in Table I after the average of the determinations for the type of sample.

It is seen that a considerable amount of the S^{35} had entered the gastrointestinal tract by 1 hr., and that the largest quantities at this time were found in the upper third of the small intestine (Fig. 1). The small amount of activity in the stomach and the lower sections of the intestine at this interval may be due to blood remaining in the tissue.

[2] The S^{35} used in this investigation had a specific activity of approximately 1 mcurie/0.05 mg. sulfate, and was supplied by the Clinton Laboratories, on allocation from the Isotopes Division, United States Atomic Energy Commission.

TABLE I

Recovery of S^{35} Following Intravenous Injection

(Per cent of injected dose)

Time interval	No. in group	Blood	Bile	Urine	Liver	Stomach	Small intestine			Large intestine	Feces	G.I. tract + feces	Total
							Upper ⅓	Middle ⅓	Lower ⅓				
Hr.													
1	4	8.8	1.3	14.6	0.4	1.2	7.4	1.3	1.1	2.6	—	13.6	44.5
3½	4	5.9	2.5(3)[a]	29.1	1.5	1.3	3.1	4.9	1.2	.9	—	10.7	50.4
6½	4	1.4	8.0(2)	62.8	1.4	0.8	2.0	3.7	2.2	1.8	—	10.5	80.6
10	6	0.6(5)	9.0(5)	66.7(5)	0.6	0.6	0.9	1.6	2.8	3.4	0.3	9.1(5)	82.4
15	4	0.2	10.4(3)	68.6(3)	0.5	1.0	0.6	0.9	1.1	2.9	1.1(2)	8.0(2)	87.1
24	3	<0.1	9.4(2)	75.3(3)	<0.1	0.4	0.3	0.3	1.1	2.4	3.9(3)	8.4(3)	93.1

[a] The percentages given are an average of the number of specimens in each group unless limited to the number in parentheses.

As time progressed, S^{35} was recovered in larger amounts from the lower portions of the small intestine and large bowel and began to appear in the feces at 10 hr. The total quantities in the gastrointestinal tract declined for 10 hr. and then leveled off. This might be attributed to absorption, or perhaps in part to the falling sulfate level of the blood. At 24 hr., approximately 4% of the injected dose had appeared in the feces, and an equal quantity was found within the large bowel and lower third of the small intestine together.

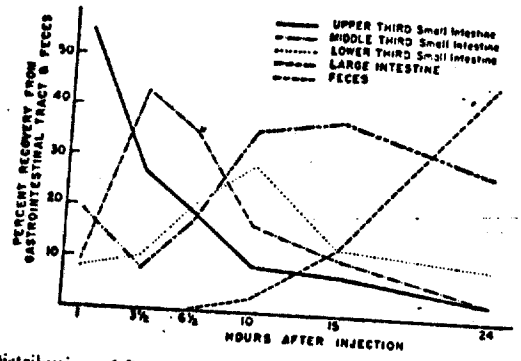

FIG. 1. Distribution of S^{35} in intestines and feces. The total recovery from the intestine and feces at each time interval is considered as 100%. See table for actual figures.

TABLE I

llowing Intravenous Injection

:t of injected dose)

rom- ach	Small intestine			Large intestine	Feces	G.I. tract + feces	Total
	Upper ⅓	Middle ⅓	Lower ⅓				
.2	7.4	1.3	1.1	2.6	—	13.6	41.5
.3	3.1	4.9	1.2	.9	—	10.7	50.1
.8	2.0	3.7	2.2	1.8	—	10.5	80.6
.6	0.9	1.6	2.8	3.4	0.3	9.1(5)	82.1
.0	0.6	0.9	1.1	2.9	1.1(2)	8.0(2)	87.1
.4	0.3	0.3	1.1	2.4	3.9(3)	8.4(3)	93.1

:age of the number of specimens in each group
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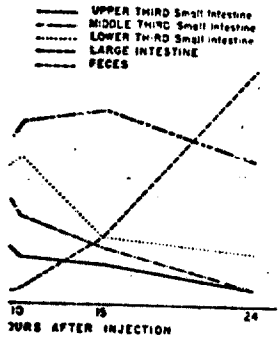

ies and feces. The total recovery from the i is considered as 100%. See table for actual

The percentages given in the table are based upon total organ weights and complete excretion samples. The total blood value was calculated on the assumption of 5 ml. of blood/100 g. of body weight. Complete bile collections were not always obtained as in some cases the collection vial broke or lost its connection with the cannula. Nevertheless, these rats were of value for other data and were hence retained in the series since the bile continued to flow and did not enter the intestine. However, S^{35} values for their bile were not determined and are not included in the data. The urine and fecal samples were found to be cross-contaminated in some of the rats sacrificed at the longer intervals. In such instances the activity of the combined samples was used for determining the total recovery of excreted S^{35}, but the data could not be obtained for urine and fecal excretion separately. The urine contained 75% of the injected dose at 24 hr. In fact most of the urinary excretion of sulfur occurred during the first 6½ hr. since 62.8% was recovered in the urine at this interval.

The biliary excretion of S^{35} was complete after 10–15 hr. At this time 10% of the injected dose was recovered from the bile.

No perceptible amount of S^{35} was recovered in the pancreas, parotid glands, or tissue fluids.

Discussion

The above results reveal that the combined excretion of S^{35} via the bile, urine, and feces was approximately equivalent to 89% of the injected dose of sulfate within 24 hr. This is considerably more than that reported by Dziewiatkowski (1) for the rat within the same period following intraperitoneal injection; however, after 120 hr. his total recovery was 95%. This difference in the excretion rate is probably due to the slower rate of absorption following the intraperitoneal administration. Of the 95% of S^{35} recovered by Dziewiatkowski after 120 hr., 85% was in the urine and 10% was in the feces. This distribution differs somewhat from that of the present report in which 75% was recovered in the urine and 18% in the combined feces, intestines, and bile. This difference in distribution can probably be explained by the fact that part of the sulfate which enters the intestine is absorbed and is excreted by the kidney. Thus, in the non-fistula rat, part of the sulfate entering the duodenum via the bile would subsequently appear in the urine. That absorption of S^{35} from the intestine does occur is indicated in the above results.

The observation that 9 or 10% of the administered dose of sulfate appeared in the bile within 10 hr. is in line with the results of Hanahan and Everett (5). They found that in the rat 5–14% of the sulfur liberated from S^{35} sodium estrone sulfate appeared in the bile within 24 hr. Their recovery of S^{35} from the feces of bile fistula animals, however, was more than that of the present study. This difference could be due to differences in the metabolism of the two compounds containing the sulfate radical. Furthermore, the higher sulfate content of the feces in the former study could have been due in part to urine contamination. In the present study the feces were removed at frequent intervals from the metabolism cages, thus minimizing the possibility for urine contamination.

Summary

Bile fistula rats given S^{35} sodium sulfate intravenously excreted 89% of the injected sulfur within 24 hr. Seventy-five per cent of the sulfur was recovered in the urine, 10% in the bile, and 4% in the feces. Determinations for sulfur were made on segments of the gastrointestinal tract at intervals after injections. The results reveal that in the bile fistula animals sulfur enters the upper third of the small intestine and moves through the intestines to be eliminated in the feces. The suggestion is made that sulfate passes directly from the blood or tissue fluid into the upper small intestine.

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THE OSMOTIC EFFECTS OF SOME SIMPLE MOLECULES AND IONS ON GASTRIC EMPTYING

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The idea that the rate of emptying of the stomach is strongly influenced by the osmotic pressure of the gastric contents stems from the experiments of Carnot & Chassevant (1905). They found that in dogs with duodenal fistulae saline nearly isosmotic with plasma left the stomach more rapidly than saline solutions which were more concentrated or more dilute. The influence of the concentration of salt on gastric emptying appears to be much the same in man as it is in dogs. Shay & Gershon-Cohen (1934) in their radiographic studies noted that tap water left the stomach more slowly than isotonic saline. They also concluded that in order to slow emptying—presumably relative to the emptying of tap water—'hypertonic' solutions of salts and glucose were necessary, whereas hydrochloric acid slowed emptying in solutions less concentrated than plasma in terms of milli-osmoles per litre.

The present experiments, extending those described by Hunt in 1956, are a study of the working of the receptors concerned in the slowing of gastric emptying that appear to be sensitive to the osmotic properties of the luminal contents of the duodenum and small intestine (Shay & Gershon-Cohen, 1934). The results of some experiments with acids which probably work differently (Sircus, 1958) are also described for comparison.

METHODS

After washing out the stomach with 250 ml. of tap water at about 8 a.m., test meals of 750 ml. of distilled water at 37° C containing various concentrations of solutes were given to four fasting volunteer medical students and the experimenters. The test meals contained, in addition to the solutes introduced as experimental variables, phenol red about 60 mg/l., which served as a marker and had no material influence on gastric emptying (Hunt, 1956). The meal was given down a tube into the stomach in about 75 sec. The tube was usually left down until the gastric contents were recovered some 10–30 min later, and the stomach was then washed out with 250 ml. tap water. The amount of phenol red recovered in this wash-out divided by the concentration of phenol red in the previously recovered major portion of gastric contents gave the volume of gastric contents not aspirated previously, usually about 20 ml. The volume of the original meal remaining in the stomach at the time of the recovery was calculated from the amount of phenol red recovered. In arranging these

experiments the interval between giving the meal and recovering it was usually adjusted for each subject so that the volume of meal recovered was at least 100 ml. out of the original 750 ml. given. Strict adherence to the routine procedure was necessary to obtain reproducibility.

The data reported here were collected during five years: because of a realization that the data initially obtained were inadequate in range or number for a new purpose, an interval of years separates some of the experiments within a single series in the same subject. Although the subjects studied are fewer than we would wish, the large number of meals given, over a thousand, and their scatter in time do provide some safeguard against the results being relevant only to special circumstances. The intensive study of a few subjects was necessary to reduce the variability due to differences between subjects, so allowing quantitative comparisons of the action of solutes and mixtures of solutes. In some subjects who gave very variable results the experiments were ended shortly and these results are not considered.

RESULTS

The results record the relations between the volume of a test meal recovered from the stomach at a fixed time after its instillation and the milliosmolar (m-osm) concentration of solutes in the instilled meal. For the most part these relationships were linear, so that straight lines could be fitted to the data by the method of least squares. Where the relationship was not linear over the whole range, for example in Fig. 4, the lines were calculated only for that part of the range which appeared on inspection to be linear. Such lines are shown as full; curved lines, shown interrupted are fitted by eye.

The influence of some monovalent and divalent anions and cations on the volume of meal remaining in the stomach after a fixed interval

Figure 1 shows the relationship between the concentration of either hydrochloric acid or potassium chloride and the volume of test meal recovered after 20 min in subject J.N.H. It may be seen that hydrochloric acid was about three times more effective per osmole than potassium chloride in increasing the volume of test meal recovered from the stomach. Similar data for other electrolytes are given in Table 1. As the concentrations were raised, so the amount of the original test meal recovered from the stomach after a fixed interval increased. Hydrochloric acid was the most effective per osmole, ammonium chloride the least effective.

In previous work (Hunt, 1956; Pathak, 1959) it was noted that the threshold concentration for the slowing of gastric emptying by hydrochloric acid was about 20 m-osm, and in the present work that for potassium chloride seemed to be at least 40 m-osm. Accordingly the regression equations shown in Table 1 for solutions of hydrochloric acid and for solutions of potassium chloride include only data for test meals containing concentrations of acid of 20 m-osm or more, and concentrations of potassium chloride of 40 m-osm or more. Reference to the intercepts (a) of the regression lines for meals containing hydrochloric acid or potassium

chloride and the axis for volume, in part 1 of Table 1, shows that in W.D.L.P. and J.N.H. the mean values for the volume of meals of water remaining at 20 min are less than those shown in part 2. This finding is consistent with the view that there is a threshold concentration for the

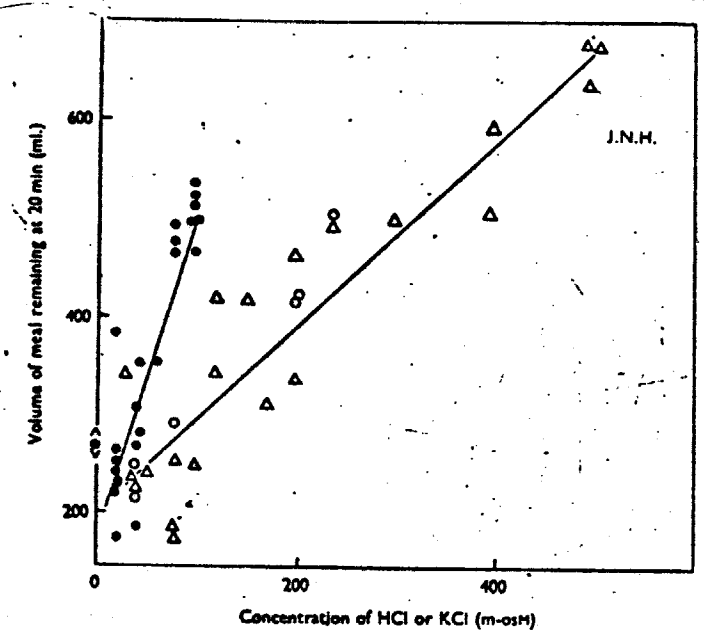

Fig. 1. A comparison of the influence of HCl (●) and of KCl (△) on the volume of test meals remaining in the stomach after 20 min.. ○ = KCl+40 m-osmoles HCl.

slowing action of hydrochloric acid and of potassium chloride. It may also be seen in Fig. 1 that potassiun chloride was equally effective in slowing gastric emptying with and without 40 m-osM hydrochloric acid.

Table 1 also shows data for solutions containing ammonium chloride, which was less effective per osmole in slowing gastric emptying than either ammonium sulphate or calcium chloride. Putting these data together the conclusion seems warranted that calcium and sulphate were effective, whereas ammonium and chloride were relatively ineffective, in slowing emptying.

The influence of sodium salts on the volume of meal recovered after a fixed interval

All the solutes so far considered except ammonium chloride increased the volume of meal recovered as the concentration of solute in the meal

was raised. Sodium salts had a different action, as may be seen from Figs. 2 and 4 for example. As the concentrations of sodium chloride, sulphate or bicarbonate (Hunt, 1956) were raised, the volumes recovered from the stomach after a fixed interval of time fell until the concentration in the meal at the time of instillation reached about 250 m-osM with the chloride or bicarbonate, and about 50 m-osM with the sulphate. Data similar to those shown in Fig. 2 for J.N.H. were obtained for J.D.P. At higher concentrations all these salts of sodium increased the volume of the gastric contents recovered as the concentration in the test meal was raised.

TABLE 1. The influence of solutions of hydrochloric acid, sulphuric acid, potassium chloride, ammonium chloride, ammonium sulphate and calcium chloride on the volume of test meals remaining in the stomach after twenty minutes

Subject ...	J.D.P.				W.D.L.P.				J.N.H.			
Range of concentrations (m-osM)	n*	a	b	s.e. of b	n	a	b	s.e. of b	n	a	b	s.e. of b
HCl 20–100	14	375	2·66	±0·67	13	163	4·17	±0·65	22	180	3·07	±0·38
KCl 40–500	16	295	1·25	±0·20	30	265	0·95	±0·13	22	200	0·93	±0·25
	Mean volumes (ml.) of water remaining at 20 min											
Water	10	252 (s.e. ±18)			11	319 (s.e. ±15·3)			19	280 (s.e. ±12·5)		
	Regression lines fitted to data including those for meals of water											
NH_4Cl 0–200	19	260	0·44	±0·16	21	323	0·20	±0·14	26	279	0·005	±0·18
$(NH_4)_2SO_4$ 0–300	19	230	0·91	±0·13	15	318	0·90	±0·18	23	273	0·64	±0·18
$CaCl_2$ 0–300	32	295	0·81	±0·14	18	327	0·86	±0·14	35	260	0·44	±0·14
H_2SO_4 0–100	14	264	4·80	±0·84	14	321	2·89	±0·50	23	242	2·60	±0·37

* y = volume of meal remaining (ml.) at 20 min; x = concentration of solute (m-osM); n = number of observations; a corresponds to the volume of meal remaining with zero concentration of solute, that is the intercept of the regression line and the ordinate; b corresponds to the slopes of the lines as shown in Fig. 1; and $y = a + bx$.

The influence of glucose and sodium chloride together on the volume of test meal recovered after a fixed interval

Glucose added to test meals increased the volume of the test meal recovered as the concentration was raised (Hunt, 1956) like the solutes referred to in Table 1. Figure 3 gives data on the effect of various concentrations of glucose, on the relation between the concentration of sodium chloride and the volume of meal recovered after 30 min. It may be seen:

(1) That 382 m-osM glucose in the meal apparently made the volume recovered independent of the concentration of sodium chloride in the meal, that is, the highest line on the figure is horizontal;

(2) That increase in the concentration of glucose increased the volume recovered from the stomach in the presence of various concentrations of sodium chloride;

(3) That the minimum volume recovered occurred with about 50 m-osM sodium chloride in the meal instead of 250 m-osM sodium chloride in the absence of glucose.

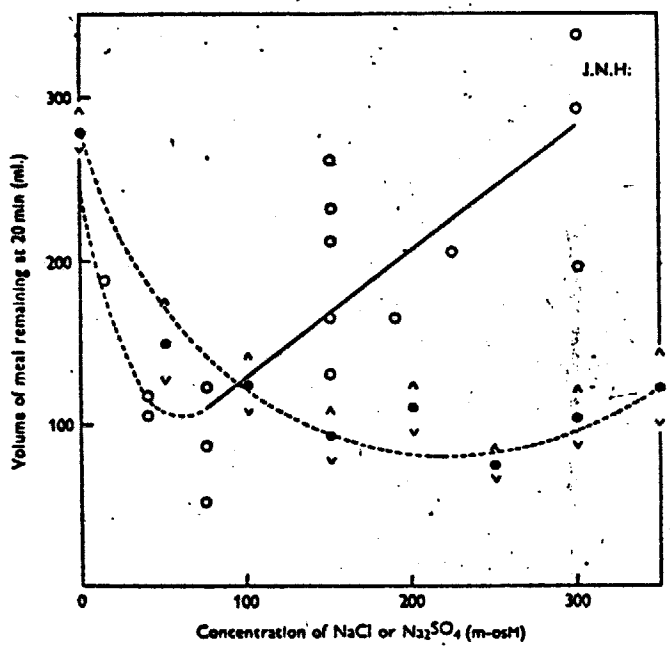

Fig. 2. A comparison of the influence of NaCl (●) and of Na_2SO_4 (○) on the volume of test meals remaining in the stomach after 20 min (J.N.H.). In all Figs. S.E. of means indicated by $\frac{\wedge}{\vee}$.

Figure 4 shows some data from experiments designed to put the conclusions from Fig. 3 on a more secure basis. In this instance the recoveries were made after 20 min and the effect of 126 m-osM glucose and sodium chloride was compared with that of sodium chloride without glucose. The figure shows:

(1) That the concentration of sodium chloride giving a minimal recovery, that is, maximal rate of emptying, is less with 126 m-osM glucose than it is with sodium chloride alone;

(2) That the increase in the volume recovered per osmole increase in concentration of sodium chloride in the meal is less in the presence of glucose;

(3) That at high concentrations of sodium chloride the volume recovered

with the meals containing glucose was *less* than with the meals containing sodium chloride alone.

Altogether 306 test meals of this type were given to three subjects. In the other two the results were similar to those shown in Fig. 4, except that in one the lowering of the concentration of sodium chloride giving the maximal recovery in the presence of glucose was not shown.

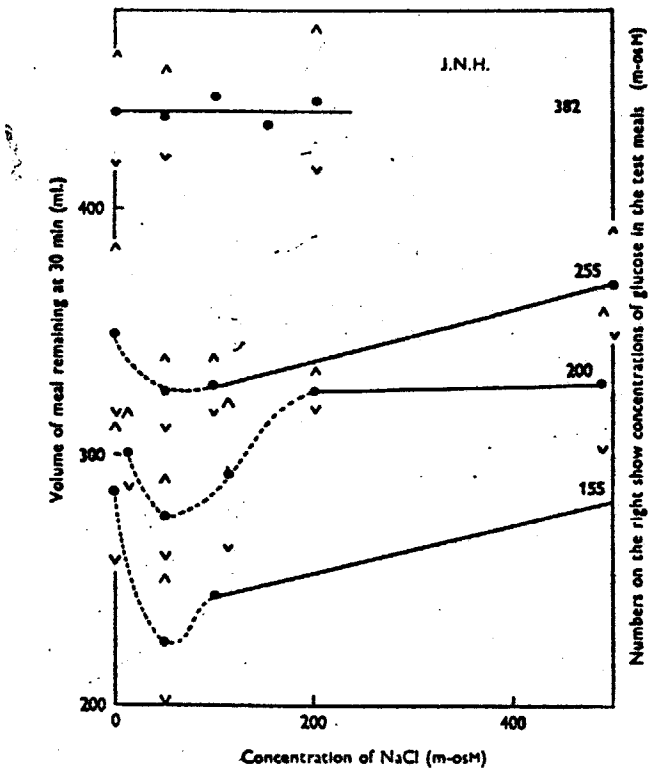

Fig. 3. The influence of different concentrations of glucose on the volume of saline solutions remaining in the stomach after 30 min (J.N.H.).

The relation between the concentration of sodium chloride and the volume of meal recovered was further studied with an experiment of the type illustrated in Fig. 3 in J.D.P. and J.N.H., with recoveries at 20 min after instillation. The slopes of the lines relating concentration of sodium chloride to volume recovered, over the range where the relationship seemed linear, are given in Table 2. The slopes became less as the concentration of

glucose was raised, as may be seen from Fig. 5, in which the slopes of the lines from Table 2 have been plotted against the relevant concentration of glucose. By extrapolation the concentration of glucose abolishing the effect of the concentration of sodium chloride on the volume of meal recovered was found to be 254 m-osm for J.N.H. and 270 m-osm for

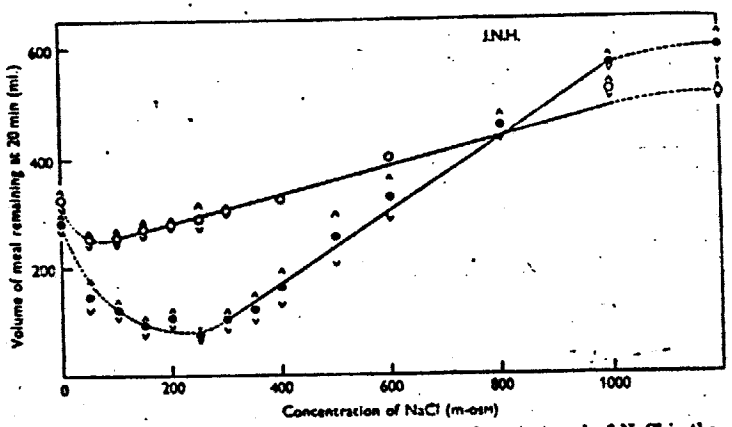

Fig. 4. The influence of the concentration of NaCl alone (●) and of NaCl in the presence of glucose 126 m-osm (○) on the volume of test meals remaining in the stomach after 20 min (J.N.H.).

TABLE 2. The influence of the concentration of glucose on the relationship between the volume of meal remaining in the stomach after twenty minutes and the concentration of sodium chloride in the test meal

Concentration of glucose (m-osm)	Range of concentrations of NaCl (m-osm)	J.D.P. n	J.D.P. a	J.D.P. b	J.D.P. S.E. of b	Range of concentrations of NaCl (m-osm)	J.N.H. n	J.N.H. a	J.N.H. b	J.N.H. S.E. of b
0	160–780	16	114	0·42	±0·07	350–1000	15	−105	0·68	±0·06
100	200–500	5	120	0·39	±0·09	150–800	7	124	0·385	±0·04
127	—	—	—	—	—	100–600	30	227	0·26	±0·0[illegible]
155	190–810	8	265	0·16	±0·07	200–810	8	298	0·22	±0·0[illegible]
200	200–800	12	370	0·17	±0·05	100–1000	10	377	0·13	±0·0[illegible]
255	—	—	—	—	—	0–1000	10	505	0·036	±0·01[illegible]

* y = volume of meal remaining at 20 min (ml.); x = concentration of sodium chloride (m-osm); n = number of observations; a corresponds to the volume of meal remaining with zero concentration of sodium chloride, that is the intercept of the regression line and the ordinate; b corresponds to the slope of the lines similar to those shown in Fig. 3; $y = a + bx$.

J.D.P. When these values are adjusted for the osmotic coefficient of glucose they become 274 and 290, that is slightly less than isosmolar with plasma.

An interesting feature of this failure of the mechanism determining gastric emptying to respond to sodium chloride in the presence of glucose

can be appreciated by looking at Fig. 4. The volume of meal recovered with 1200 m-osm sodium chloride was *less* in the presence of 126 m-osm glucose than it was without the glucose, although in the absence of sodium chloride glucose *increased* the amount recovered. It seemed desirable to make a direct contrast between the influence of a constant concentration of glucose upon the response to a changing concentration of sodium chloride, and the influence of a constant concentration of sodium chloride

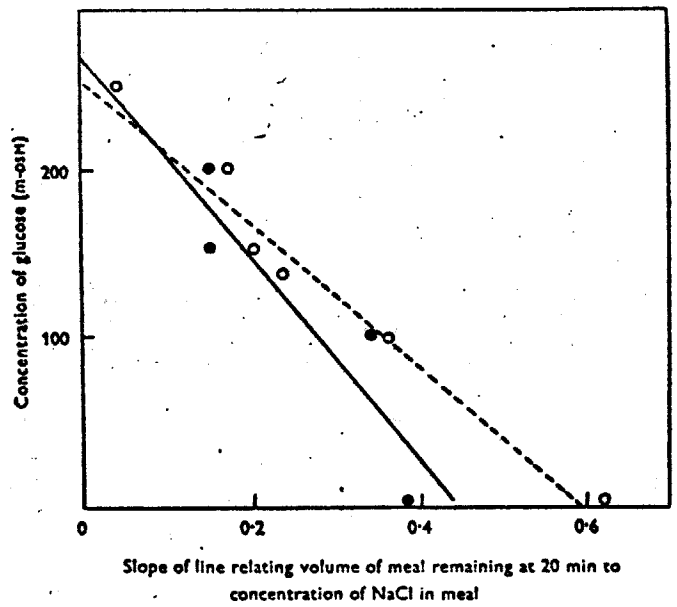

Fig. 5. The relation between the concentration of glucose in a test meal and the effect of NaCl on the volume of test meal remaining in the stomach after 20 min. ● J.D.P.; ○, J.N.H.

upon the response to a changing concentration of glucose, although the general form of the relationship at concentrations of sodium chloride up to 200 m-osm is apparent from Fig. 3. Eight test meals of 30 min duration, containing varying amounts of glucose and 500 m-osm sodium chloride, were given to J.N.H.: the slope of the line relating concentration of glucose to volume of meal recovered was 1·27, S.E. ± 0·27. There is thus no doubt that sodium chloride even at a m-osm concentration greater than that of the plasma did not abolish the slowing action of glucose on gastric emptying, whereas a solution of glucose isosmotic with plasma did abolish the slowing action of sodium chloride.

The influence of sodium chloride on gastric emptying in the presence of acid

Figure 6 shows the relationship based on 132 meals in three subjects between the concentration of sodium chloride in test meals and the volume

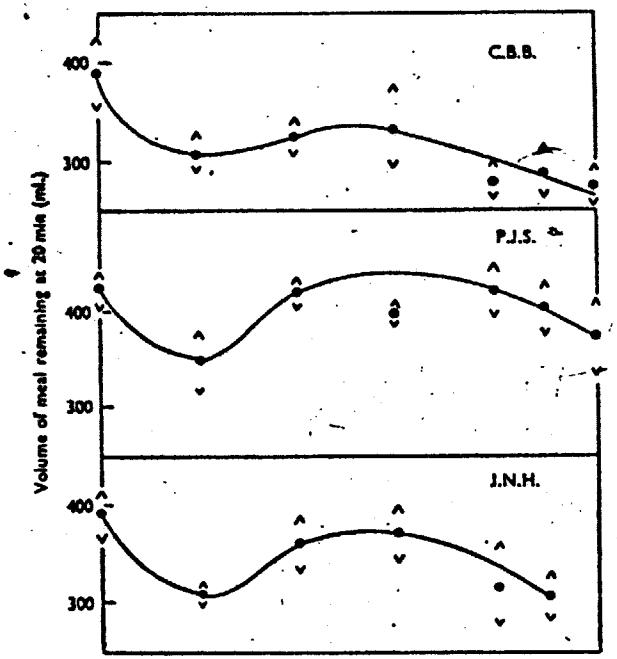

Fig. 6

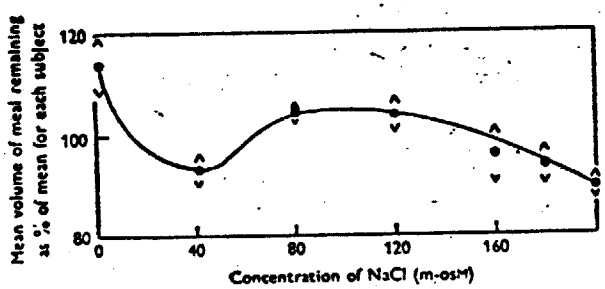

Fig. 6a

Fig. 6. The influence of sodium chloride in the presence of 40 m-osM HCl on the volume of test meals remaining in the stomach after 20 min (C.B.B., P.J.S. and J.N.H.); (6a) combined data of Fig. 6.

of meal recovered in the presence of 40 m-osM hydrochloric acid. These data were combined by expressing the volume of each recovery as a percentage of the mean volume of all recoveries for that subject. The means of these percentages for the three subjects are shown at the foot of Fig. 6. In the presence of 40 m-osM hydrochloric acid there was a lower recovery with the meal containing 40 m-osM sodium chloride than with immediately higher or lower concentrations of sodium chloride. With higher concentrations of sodium chloride the volume recovered fell as the concentration of sodium chloride was raised to 200 m-osM. It was not possible to explore the effects of higher concentrations of sodium chloride because they produced nausea.

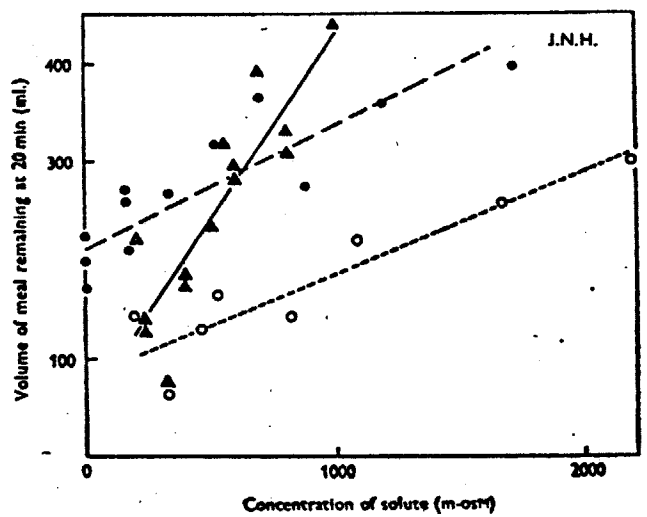

Fig. 7. The influence of ethanol (●), urea (▲) and glycerol (○) on the volume of test meals remaining in the stomach after 20 min (J.N.H.).

The influence of urea, ethanol and glycerol on the volume of test meal recovered after a fixed interval

Figure 7 shows data obtained in J.N.H. from test meals containing urea or glycerol. The response to test meals containing these solutes was similar to the response to meals containing sodium salts. There was a minimal recovery with 100-200 m-osM solutions. At higher concentrations these solutes gave progressively larger recoveries. Figure 7 also shows that with meals containing ethanol no minimal recovery was detected, the volume recovered increasing progressively as the concentration of ethanol in the meal was increased from zero.

DISCUSSION

The present experiments have confirmed the conclusions of earlier workers (Apperly, 1926; McSwiney & Spurrell, 1933; Shay, 1944) that there is a relationship between the concentration of a solute in a test meal and the rate of gastric emptying of the meal, and that this relationship varies with the solute concerned. The problem here is to describe the working of the receptor mechanism in this regulation of gastric emptying.

The rate of emptying of the stomach might be thought to depend upon the degree of distension of the duodenum or jejunum by the gastric effluent. If the original meal contained some solute which was very little absorbed, the volume of the contents of the sensitive segment might remain large as a result of the osmotic action of the solute, and gastric emptying would be slowed. Hypotheses of this kind appear to be made untenable by the equal action per osmole of calcium chloride and sulphates (Ingraham & Vissher, 1938), which are relatively little absorbed from the intestine, and glucose which is very readily absorbed. In addition, it has been found that when two meals are given in succession the second, passing into a full intestine, leaves the stomach more rapidly than the first (Hunt & Macdonald, 1954). Further, with meals of 750 ml. the 'half-life' of the emptying process is constant over the period during which the main part of the meal empties, but the fullness of the intestine must vary considerably during this time (Hunt & Macdonald, 1954). Taken together these facts suggest that some mechanism other than the fullness of the duodenum or small intestine controls gastric emptying under these conditions.

In the discussion which follows, the stimulus to the receptor mechanism has to be assumed to be adequately described by specifying the composition of the test meal. The experiments are designed to minimize the importance of any alimentary contributions to the environment of the receptor by washing out the stomach before the meal is given, by using a large volume of meal and by considering data mainly obtained from meals of short duration. At the time of the recovery the meal was usually diluted by about 10% as judged from the change in concentration of phenol red. There was no evidence that the highly concentrated solutions were more diluted in the stomach (Hunt, Macdonald & Spurrell, 1951), the greatest dilution occurring with meals which emptied rapidly, because of the smaller volume of the meal diluted by secretion and because secretion was less inhibited. At the time of the recovery the concentration in the gastric contents of acid derived from secretion was almost always less than 10 m-equiv/l., which is below the known threshold for effects on gastric emptying: and, finally, some of the receptors responding to solutes in the gastric contents probably lie just beyond the pylorus (Shay & Gershon-

Cohen, 1934), so that dilution by pancreatic secretion or bile might not influence the solution in contact with them. Considering all these points together, it may be concluded that although assessing the stimulus to the receptors from the composition of the instilled meal introduces an error it can be accepted for the moment. None of the test meals used in the experiments reported here produced nausea. Even had they done so it would not invalidate the results, since the work is concerned with the relationship between stimulus, receptors and response and not with the pathways mediating the response. It appears that the receptors of low threshold initiating the sensation of nausea lie in the walls of the duodenum (references cited by Hunt, 1956).

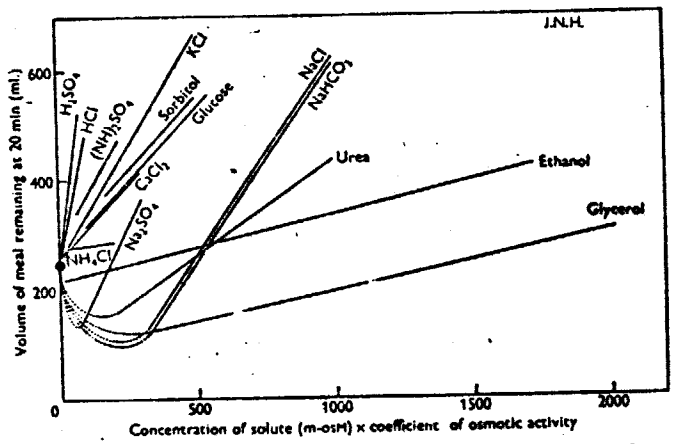

Fig. 8. The influence of the osmotic activity of various solutes on the volume of test-meal remaining in the stomach after 20 min (J.N.H.). Because Table 1 and Fig. 1, 2 and 4 include divergent data for eight meals of water obtained after the majority of the experiments in Fig. 8 were completed, the mean volume of meal of water recovered is not the same in Fig. 8 as in the other Figures. (On figure, for $(NH)_2SO_4$ read $(NH_4)_2SO_4$.)

It may be seen in Fig. 8 and Table 3 that the slopes of the lines relating the m-osM concentration of solute and the change in volume of meal recovered are approximately the same for ammonium sulphate, potassium chloride, sorbitol, glucose, calcium chloride and at high concentrations for sodium chloride, sodium sulphate and sodium bicarbonate. These compounds include combinations of monovalent and divalent anions and cations, and non-electrolytes, and it is difficult to envisage a separate receptor for each. On the other hand a single receptor responding to all more or less equally would presumably be sensitive to some common property: their osmotic activity is a candidate for consideration and there

is no obvious alternative. Hydrochloric and sulphuric acids are much more potent than the solutes mentioned above, so that they probably work via a different mechanism as they do in controlling gastric secretion in the dog (Sircus, 1958). It will be assumed that the osmotic regulation of emptying depends upon a single type of receptor, although at the moment there are no grounds, apart from the principle of Occam's razor *entia non sunt multiplicanda praeter necessitatem*, for supposing that this is so.

TABLE 3. Relative activity of solutes on the osmoreceptor mechanism in one subject

Solute	a*	b	S.E. of b	n	Range of concentrations (m-osm)
H_2SO_4	242	2·60	±0·37	16	0–77
HCl	238	2·32	±0·11	43	0–100
HCl	180	3·07	±0·38	22	20–100
KCl	200	0·93	±0·25	21	40–500
KCl	257	0·75	±0·07	40	0–500
$(NH_4)_2SO_4$	279	0·64	±0·18	23	0–225
Glucose	260	0·57	±0·04	36	0–550
Sorbitol	281	0·50	±0·07	24	0–500
$CaCl_2$	260	0·44	±0·14	35	0–300
Ethanol	213	0·12	±0·02	12	0–1720
NH_4Cl	273	0·005	±0·18	26	0–200
Na_2SO_4	52	0·77	±0·26	13	75–300
$NaHCO_3$	−115	0·70	±0·11	12	320–1000
NaCl	−105	0·68	±0·06	15	350–1000
Urea	76	0·38	±0·10	14	200–1000
Glycerol	85	0·10	±0·02	8	330–2170

* y = volume (ml.) of 750 ml. meal remaining in the stomach 20 min after ingestion; x = concentration of solute (m-osm); n = number of observations; $y = a + bx$; a corresponds to the volume (ml.) of meal remaining at zero concentration of solute, that is the intercept of the regression line with the ordinate.

So far the effects of solutes on gastric emptying can be described and explained in seven sections which may be more readily understood when they are considered in conjunction with Table 3 and Fig. 8.

(1) Glucose, sorbitol, sulphates, salts of potassium, calcium and at high concentrations only, sodium, have about equal effects per osmole in slowing gastric emptying. They are postulated to reduce the volume of sensitive component of an osmoreceptor responding to the contents of the alimentary lumen beyond the pylorus. These solutes are thought to penetrate the selective membrane of the osmoreceptor slowly if at all. A signal from the osmoreceptor which slows gastric emptying is postulated to occur with test meals of pure water and to increase when the flux of water into the sensitive component is reduced by the osmotic action of solutes in the lumen.

(2) Ammonium, bicarbonate and chloride are postulated to penetrate the osmoreceptor freely by diffusion and to have no effect on gastric emptying except the inhibition by the water in which they are dissolved.

However, when any one of these ions is paired with any ion listed under 1, it is restrained from penetrating by the demands of electrical neutrality and has an action per osmole equal to any solute listed under 1. There is evidence to suggest that bicarbonate may penetrate more freely than chloride (Hunt, 1956).

(3) Ethanol and, at high concentrations, urea and glycerol, are postulated to penetrate the osmoreceptor more slowly than unrestrained chloride, bicarbonate or ammonium but more rapidly than the solutes listed under 1. As these non-electrolytes continue to have an action on the hypothetical osmoreceptor for up to 20 min, their steady-state distribution across the osmoreceptor membrane presumably sets up an osmotic gradient greater than that postulated for ammonium and bicarbonate, which reduces the flux of water into the receptor.

(4) At low concentrations sodium, urea and glycerol are postulated to be subject to either facilitated diffusion or active transport into the osmoreceptor, thus favouring the flux of water into the receptor and reducing the inhibitory action of the receptor which is postulated to occur with pure water.

(5) Solutions of glucose isosmotic with plasma are postulated to make the membrane of the osmoreceptor so permeable to sodium that it neither osmotically reduces the volume of the osmoreceptor at high concentrations nor increases it at low concentrations as a result of its supposed facilitated migration into the receptor.

(6) Strong acids and glucose are postulated to have different modes of action since hydrochloric acid and glucose have different effects on the response of the osmoreceptor to sodium chloride. Moreover, strong acids are about three times more effective per osmole than the next most powerful solutes in slowing gastric emptying. These views are in harmony with those of Sircus (1958) on the control of gastric secretion.

(7) The increased rate of emptying with low concentrations of sodium sulphate may possibly be the result of the presence of bicarbonate or chloride ion secreted from the pancreas. At higher concentrations of sulphate the effect of the chloride may be masked.

In devising further studies of the supposed osmoreceptor the red cell, which has been studied as an osmometer by Ponder (1940), may provide a useful model. Many of the properties postulated for the osmoreceptor have been demonstrated for red cells: for example, facilitated migration of glycerol (LeFevre, 1948) and sodium (Davson & Reiner, 1942); increased permeability to sodium on exposure to hypertonic solutions (Davson & Reiner, 1942); penetration by ammonium (Jacobs & Parpart, 1938); slower penetration by sulphate than by chloride (Davson, 1959, from data of Luckner, 1948) and restraint of penetration of chloride and bicarbonate

by potassium, sodium or calcium (Ponder, 1953) are accepted. At high concentrations the red-cell membrane behaves as though it were impermeable to glucose (Widdas, 1954). It is interesting that the very indirect approach via studies of gastric emptying can lead to postulates so similar to the known facts about red cells.

SUMMARY

1. The influence on gastric emptying of several solutes in test meals was studied by plotting the volume of test meal remaining after a fixed interval against the osmolar concentration of the solute in the original meal.

2. On an osmolar basis hydrochloric acid and sulphuric acid were about three times as potent as potassium chloride in slowing gastric emptying.

3. Ammonium sulphate and calcium chloride were two to four times more potent than ammonium chloride in slowing gastric emptying.

4. At low concentrations the addition of sodium chloride and sodium sulphate to test meals increased the rate of gastric emptying. The osmolar concentration in the meal giving a maximal rate of emptying was less for sodium sulphate than for sodium chloride. At high concentrations sodium sulphate and sodium chloride were about equally potent in slowing gastric emptying.

5. Increasing the concentration of glucose in test meals containing sodium chloride progressively reduced the action of sodium chloride on gastric emptying. At concentrations of glucose nearly isosmotic with plasma sodium chloride lost all its influence on gastric emptying. In contradistinction, solutions of sodium chloride isosmotic with plasma did not abolish the inhibitory action of glucose on gastric emptying.

6. In the presence of 40 m-osm hydrochloric acid low concentrations of sodium chloride increased the rate of gastric emptying as they did in the presence of equipotent concentrations of dextrose. A complex relationship between concentration of sodium chloride and the rate of gastric emptying in the presence of acid was defined.

7. Urea and glycerol in test meals hastened gastric emptying at low concentrations but slowed it at high concentrations. Ethanol slowed gastric emptying as the concentration was increased.

8. The findings are explained on the assumption that there is an osmoreceptor mechanism in the intestine which inhibits gastric emptying. The majority of solutes are assumed not to penetrate the receptor, but sodium, urea and glycerol are thought to be subject to facilitated transport across the osmotic membrane at low concentrations and urea and glycerol to slow diffusion at high concentrations. Solutions of glucose isosmotic with plasma are thought to make the supposed osmoreceptor permeable

to sodium. The action of acids is thought to depend upon a separate mechanism.

We are greatly indebted to the students who acted as subjects in this work, without whose unfailing co-operation it would have been impossible. It is a pleasure to acknowledge help received in discussion with Drs H. Davson and W. F. Widdas. We are very grateful to Professor W. R. Spurrell for much improving the manuscript.

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INORGANIC SULPHATE, SULPHITE AND SULPHIDE AS SULPHUR DONORS IN THE BIOSYNTHESIS OF SULPHUR AMINO ACIDS IN GERM-FREE AND CONVENTIONAL RATS*

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SUMMARY

1. Incorporation of ^{35}S from ^{35}S-labelled sodium sulphate, sulphite and sulphide into the sulphur-containing amino acids cysteine/cystine and methionine was investigated in young germ-free and conventional rats. The animals were killed 3 h after intraperitoneal injection of the labelled compound.

2. After injection of [^{35}S]sulphate or [^{35}S]sulphite both cysteine and methionine were labelled slightly in conventional rats, whereas no labelling of sulphur amino acids was detectable in germ-free animals.

3. After injection of [^{35}S]sulphide, cysteine was labelled to approximately the same extent in both conventional and germ-free rats. Labelling of methionine in conventional animals was much smaller and in germ-free rats not detectable.

4. These findings prove that inorganic sulphur at the sulphide level of oxidation can be used in rat tissues for the synthesis of cysteine by a mechanism not involving methionine as the ultimate sulphur donor, most likely by enzymic sulphhydrylation of serine. The complete lack of incorporation of ^{35}S from labelled sulphate and sulphite into cysteine in germ-free animals indicates that these compounds are not reduced to sulphide in noteworthy amounts in rat tissues. The intestinal microorganisms are thus responsible for the utilization of sulphate and sulphite in the sulphur amino acid synthesis observed in conventional rats.

INTRODUCTION

The well known route for cysteine formation in animal tissues leads from methionine *via* the intermediates homocysteine and cystathionine to cysteine[1,2]. Animals which characteristically are unable to synthesize the carbon chain of methionine are dependent on the dietary supply of this sulphur-containing amino acid. If sufficient methionine is fed, cysteine is no longer needed in the diet[3].

In plants and many microorganisms inorganic sulphur is fixed primarily into organic linkage as cysteine and transferred to a different carbon chain for the synthesis

* A report of this work was presented at the XII Scandinavian Congress of Physiology, August 24th, 1966 in Turku (Finland)[20].

Self

of methionine. The general reaction scheme in sulphur fixation by microorganisms involves the genetically controlled stepwise reduction of activated sulphate (3'-phosphoadenosine-5'-phosphosulphate) *via* intermediate sulphite formation to inorganic sulphide[4–7]. For the final step in sulphur fixation, formation of cysteine, two mechanisms have been proposed: (a) the reversal of the cysteine desulphhydrase (L-cysteine hydrogensulphide-lyase (deaminating), EC 4.4.1.1) reaction[8]: L-cysteine + $H_2O \rightleftharpoons$ pyruvate + NH_3 + H_2S; (b) the cysteine synthase (L-serine hydro-lyase (adding H_2S), EC 4.2.1.22) reaction[9]: L-serine + $H_2S \rightleftharpoons$ L-cysteine + H_2O. Both enzymes are present in mammalian tissues, too[8,10].

There is no evidence of any appreciable sulphate reduction in mammalian tissues. However, incorporation of ^{35}S from labelled sulphate into sulphur-containing amino acids *in vivo* has been reported by several authors (for literature see ref. 11) and commonly attributed to the metabolic activity of the intestinal flora. Parenterally administered inorganic sulphur compounds are very soon recovered in appreciable amounts in the intestine and so readily subjected to the activities of various intestinal microorganisms. This seriously interferes with experiments made with conventional animals.

The use of germ-free animals allows an approach free from interfering factors of this kind for studies on sulphur metabolism *in vivo*. In the present investigation the utilization of inorganic sulphur at various levels of oxidation for the biosynthesis of sulphur amino acids cysteine/cystine and methionine in germ-free and conventional rats was studied.

MATERIALS AND METHODS

Experimental animals

Germ-free rats of the Swedish strain reared according to GUSTAFSSON[12,13] were used. The conventional rats were of the same strain and age and reared in the animal room. Both groups of rats were fed on the semisynthetic diet D7 (see ref. 13) and water. The diet was sterilized by autoclaving at 121° for 20 min. The animals weighed 90–120 g and were of both sexes. They were kept fasting overnight before injections.

Radioactive sulphur compounds

$Na_2{}^{35}SO_4$ (carrier-free, aqueous solution), $Na_2{}^{35}SO_3$ and $Na_2{}^{35}S$ (both freeze-dried solids, sealed under N_2 atmosphere, specific radioactivities 8 mC/mmole) were purchased from the Radiochemical Centre, Amersham, England. All compounds were stored in the dark at −20° until used.

Sterile solutions containing 300 μC/ml with specific radioactivities of 8 mC per mmole were prepared from each compound for injections. $Na_2{}^{35}SO_3$ and $Na_2{}^{35}S$ were dissolved immediately before use in the original ampoules with an appropriate amount of sterilized 0.27 M sodium phosphate buffer (pH 7.2). Manipulations were carried out in a sterile isolator. The specific radioactivity of $Na_2{}^{35}SO_4$ was adjusted by adding carrier sulphate. The sulphate solution was sterilized before use by autoclaving at 121° for 20 min. To avoid losses of radioactivity from the sulphide solution due to formation of volatile $H_2{}^{35}S$, the slight shift towards a more alkaline pH (7.7) of buffer on dissolution of $Na_2{}^{35}S$ was not compensated.

After injections, the isotope solutions were made alkaline with NaOH and stored in sealed ampoules at —20° until they were checked for radioactivity.

Experimental technique

Each labelled compound was injected intraperitoneally in a volume of 1 ml into 5 germ-free and 5 conventional rats. The dose of sulphate and sulphite was 300 μC for each animal. The same dose of sulphide (2.9 mg Na_2S) was toxic and the dose of 60 μC was therefore adopted. It was well tolerated by both germ-free and conventional animals.

The rats were killed by exsanguination under ether anaesthesia 3 h after injections. The blood was collected into paraffinized tubes and the serum separated. Liver, kidneys, pancreas, stomach and the whole intestine with their contents were placed separately in plastic vials and immediately cooled with solid CO_2. Sera and organs were stored at —20° until analysed.

Total radioactivity of the gastrointestinal tract

Stomach and intestine with contents were homogenized together in 75 ml of ice-cold saline in an Ultra-Turrax homogenizer. Nine ml of 0.1 M NaOH was added to 1 ml of homogenate and the solution was kept on boiling-water bath for 30 min and filtered. Aliquots of 50 μl were applied evenly on strips of filter paper (Whatman No. 1, 38 mm × 15 mm). The dried strips were placed as cylinders in glass counting vials and 10 ml of scintillator (toluene containing 0.4 % (w/v) of 2,5-diphenyloxazole and 0.01 % (w/v) of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene) was added. After cooling in the dark the radioactivities were counted in a Packard Tri-Carb liquid scintillation spectrometer. To correct for quenching, the same homogenates mixed with a known amount of $Na_2{}^{35}SO_4$ as internal standard were counted in a similar way.

Preparation and analysis of other tissues

From liver, kidneys and pancreas 10 % (wet wt./v) homogenates were made in water in a Potter–Elvehjem type homogenizer fitted with Teflon pestle. The protein was determined according to LOWRY *et al.*[14]. Bovine serum albumin was used as standard.

To minimize losses of sulphur amino acids during the analytical procedure, quantitative oxidation of free and protein-bound cysteine/cystine and methionine was first effected. Cysteic acid and methionine sulphone formed by oxidation are stable and more suitable for isolation and quantitation[15]. Six ml of ice-cold performic acid reagent[16] was mixed with 1 ml of tissue homogenate or with 0.3–0.5 ml of serum. The mixture was kept on an ice bath for 16 h to ensure complete oxidation of sulphur compounds. Most of the performic acid was removed under reduced pressure at room temperature, the residue was hydrolysed in 6 ml of 6 M HCl at 100° for 24 h and the solids were removed by filtration. Total radioactivities of hydrolysates were measured and corrected for quenching as described above. The total radioactivities of the corresponding tissues were then calculated.

For removing most of inorganic sulphate, 1 ml of 1 M H_2SO_4 and 1 ml of 1 M $BaCl_2$ was added to each filtrate. After mixing and standing at 4° for 4 h the supernatant solution was filtered and evaporated to dryness at 40 ° *in vacuo*. The residue was dissolved in 5 ml of performic acid and the reagent removed under reduced pressure

at room temperature. The dry residue was dissolved in 0.1–0.5 ml of 0.1 M HCl. Aliquots of 10–50 μl were immediately subjected to paper electrophoresis for the separation and quantitation of cysteic acid and methionine sulphone. Their radioactivities were counted from duplicate strips by liquid scintillation[15].

RESULTS AND DISCUSSION

No marked differences were found between the distribution and intestinal excretion of the labelled sulphur compounds in germ-free and conventional animals (Tables I and II). Major effects caused by disparities between compounds in their

TABLE I

DISTRIBUTION OF RADIOACTIVITY TO SOME ORGANS OF GERM-FREE AND CONVENTIONAL RATS 3 h AFTER INTRAPERITONEAL INJECTION OF ^{35}S-LABELLED SODIUM SULPHATE, SULPHITE AND SULPHIDE

Total radioactivities in disintegrations/min per mg of tissue protein. Mean values ± standard deviation.

Group	*Number of animals*	*Dose (μC)*	*Total radioactivity*			
			Serum	*Liver*	*Kidney*	*Pancreas*
Sulphate						
germ-free	5	300	41 700 ± 4700	39 100 ± 5100	43 300 ± 4200	15 600 ± 1900
conventional	5	300	42 500 ± 4200	47 300 ± 6300	47 800 ± 3400	16 900 ± 2100
Sulphite						
germ-free	5	300	82 400 ± 6900	49 100 ± 6900	61 200 ± 3400	21 800 ± 2500
conventional	5	300	72 300 ± 7400	46 500 ± 3700	52 500 ± 4800	22 100 ± 1900
Sulphide						
germ-free	5	60	11 500 ± 1100	11 700 ± 1000	10 400 ± 1300	6 900 ± 630
conventional	5	60	8 800 ± 1000	9 700 ± 1100	8 100 ± 1400	4 500 ± 710
conventional	2	300	39 300 ± 2900	38 000 ± 3200	30 600 ± 1900	15 500 ± 1100

TABLE II

RECOVERY OF THE RADIOACTIVITY FROM ^{35}S-LABELLED SODIUM SULPHATE, SULPHITE AND SULPHIDE IN SOME ORGANS OF GERM-FREE AND CONVENTIONAL RATS 3 H AFTER INTRAPERITONEAL INJECTION OF THE LABELLED COMPOUND

Total radioactivities of whole organs in per cent of injected radioactivity. Total serum volume calculated as 3.5 ml/100 g of body weight.

Group	*Number of animals*	*Dose (μC)*	*% of injected radioactivity*				
			Serum	*Liver*	*Kidneys*	*Pancreas*	*Stomach plus intestine*
Sulphate							
germ-free	5	300	1.40	3.80	0.74	0.07	17.7
conventional	5	300	1.17	4.62	0.80	0.11	13.2
Sulphite							
germ-free	5	300	1.90	2.40	0.92	0.13	14.8
conventional	5	300	1.74	2.06	0.88	0.16	11.7
Sulphide							
germ-free	5	60	1.12	2.36	0.70	0.16	23.3
conventional	5	60	0.89	2.18	0.68	0.18	21.3
conventional	2	300	1.24	3.50	0.78	0.20	22.4

TABLE III

INCORPORATION OF ^{35}S INTO CYSTEINE/CYSTINE AND METHIONINE IN SOME ORGANS OF GERM-FREE AND CONVENTIONAL RATS 3 h AFTER INTRAPERITONEAL INJECTION OF ^{35}S-LABELLED SODIUM SULPHATE, SULPHITE AND SULPHIDE

Specific radioactivities in disintegrations/min per μmole of amino acid. Mean values ± standard deviation.

TABLE III

INCORPORATION OF ^{35}S INTO CYSTEINE/CYSTINE AND METHIONINE IN SOME ORGANS OF GERM-FREE AND CONVENTIONAL RATS 3 h AFTER INTRAPERITONEAL INJECTION OF ^{35}S-LABELLED SODIUM SULPHATE, SULPHITE AND SULPHIDE

Specific radioactivities in disintegrations/min per μmole of amino acid. Mean values ± standard deviation.

Group	*Number of animals*	*Dose (μC)*	*Specific radioactivity*							
			Serum		*Liver*		*Kidney*		*Pancreas*	
			Cys	*Met*	*Cys*	*Met*	*Cys*	*Met*	*Cys*	*Met*
Sulphate										
germ-free	5	300	0	0	0	0	0	0	0	0
conventional	5	300	440 ± 65	182 ± 8	327 ± 23	192 ± 25	188 ± 19	133 ± 11	264 ± 30	181 ± 19
Sulphite										
germ-free	5	300	0	0	0	0	0	0	0	0
conventional	5	300	333 ± 21	181 ± 17	293 ± 23	165 ± 17	182 ± 17	116 ± 15	194 ± 21	133 ± 10
Sulphide										
germ-free	5	60	794 ± 76	0	667 ± 58	0	315 ± 21	0	505 ± 30	0
conventional	5	60	872 ± 63	traces*	789 ± 70	traces*	370 ± 15	traces*	572 ± 42	traces*
conventional	2	300	3715 ± 424	190 ± 23	3146 ± 241	179 ± 12	2067 ± 158	131 ± 11	2537 ± 194	165 ± 10

* Less than 100 disintegrations/min per μmole.

absorption and distribution to organs can thus be excluded. The radioactivities of the gastrointestinal tracts tended to be slightly higher in germ-free animals and sulphite was apparently bound somewhat more firmly to serum proteins than sulphate and sulphide. The total radioactivities recovered from the gastrointestinal tracts are in good agreement with the values given by WALDSCHMIDT[11] for sulphate, according to which 10 % of injected sulphate was recovered 30 min after administration from the intestinal contents of the rat.

The specific radioactivities of the isolated sulphur amino acids in the different test groups are presented in Table III. In the germ-free animals ^{35}S only from sulphide was found in amino acid linkage in cysteine. No labelling of methionine was found in any germ-free group. In conventional animals a detectable labelling of both cysteine and methionine was found in all 3 groups. Sulphide was by far the most effective sulphur donor for the synthesis of cysteine in conventional rats whereas no significant differences were found in the utilization of the 3 sulphur compounds for the synthesis of methionine in the same animals.

According to these findings inorganic sulphate and sulphite are not utilized as sulphur donors for the synthesis of sulphur amino acids in the germ-free rat. It is of considerable interest, however, that inorganic sulphide can be fixed into an organic linkage as cysteine in rat tissues. Evidently no noteworthy reduction of sulphate or sulphite to sulphide occurs in rat tissues.

Results obtained in some preliminary experiments *in vitro* were consistent with these findings. No incorporation of ^{35}S into sulphur-containing amino acids was detectable in homogenates of germ-free rat liver, kidney and gut when incubated with labelled sulphate or sulphite. Labelling of cysteine was found in all homogenates after incubation with [^{35}S]sulphide.

At the present time the most plausible mechanism for sulphide fixation is direct sulphhydrylation of serine catalysed by cysteine synthase. This enzyme has been shown to operate in several mammalian tissues *in vitro*[10], although the favoured direction of this reversible reaction *in vivo* is not known. Fixation of sulphide by the reversal of the cysteine desulphhydrase reaction seems less likely because under physiological conditions the equilibrium lies definitely towards desulphuration of cysteine[17]. The function of this enzyme *in vivo* is in fact not established[18].

In rat tissues the sulphide fixation appears to be of minor importance quantitatively according to the low specific radioactivities of cysteine isolated from animals injected with $Na_2{}^{35}S$. Approx. 5 % of the total ^{35}S activity of visceral organs was found incorporated into cysteine.

No quantitative data concerning endogenous and intestinal sulphide formation are available. When given parenterally in large doses the major fate of sulphide is probably oxidation to sulphate and elementary sulphur[19]. Dietary conditions presumably have an effect on the fate of sulphide, too. The diet used in these experiments had a high casein content (22 %) and formation of cysteine *de novo* may have been inhibited by the availability of sulphur amino acids in excess from the food.

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MECHANISM OF THE LAXATIVE EFFECT OF SODIUM SULFATE, SODIUM CYCLAMATE AND CALCIUM CYCLAMATE (1)

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In 1950 FITZHUGH and his associates (1, 2) reported their observation of diarrhea in the rat on a diet containing 5 % sodium cyclamate (N-cyclohexyl-sulfamate). Occasional diarrhea was also noted in the rat on a diet containing 2 % or more of sodium cyclamate (3). Dogs had watery diarrhea after oral ingestion of sodium cyclamate of 2 to 4 g/kg of body weight per day (3). Human subjects who ingested calcium cyclamate at a dose of 5 g/day had some increase in bulk and moisture of the stool but no change in the frequency of passage (4). A later study in a large group of healthy human subjects revealed similar changes in the stool but only when the ingestion of the cyclamate was raised to 10 or 12 g/day (5).

Since cyclamate had no demonstrable pharmacodynamic action it was suggested that the laxative effect of large doses of sodium cyclamate might be due to an inert salt effect in a manner similar to that produced by sodium sulfate (3). The laxative effect of sodium sulfate is well known and generally ascribed to its poor absorption and osmotic retention of fluid in the gastrointestinal tract (6–9). Although many publications have appeared concerning the movement and/or osmotic effect of sodium sulfate in the alimentary canal (10–18) there are no appropriate quantitative data concerning its action in intact animals at cathartic doses.

This paper presents experiments which have been performed in

(1) An abstract presenting part of this work appeared in *Fed. Proc.*, 1956, *15*, 441.

animals to ascertain the mode of action and relative potency of the laxative effect of sodium and calcium cyclamates in comparison with sodium sulfate. The experiments were designed to assess systemic as well as local intraluminal effects of these salts on the alimentary tract. To assay the potency of different salts following oral doses it was deemed necessary to determine the relative amount that remains unabsorbed in the gut in order to correlate the laxative effect with the "dose" that acts upon the specific organ or receptors. Special attention also was given to the bulk-retaining osmotic effect of the salts, which is generally accepted as the mode of action of sodium sulfate in producing catharsis.

MATERIALS AND METHODS

The animal experiments were conducted in a number of specific but interrelated studies in several species including healthy adult albino rats (Sprague-Dawley), albino mice (Swiss) and mongrel dogs. In some experiments albino rabbits (New Zealand) and monkeys (Rhesus) were also used. All animals were kept in air conditioned quarters. Rats, mice and rabbits were maintained on the respective Rockland pellets, dogs on Ideal (Wilson) and monkeys on Purina diet. Equimolar doses of sodium cyclamate ($C_6H_{11}NHSO_3Na$, mol wt 201.23), calcium cyclamate ($(C_6H_{11}NHSO_3)_2Ca \cdot 2H_2O$, mol wt 432.58) and sodium sulfate (Na_2SO_4, mol wt 142.06) were used in most of the experiments.

When considered necessary, analytical determinations were made with sodium cyclamate-S^{35} solutions (0.6 to 2.1 × 10^6 counts/minute/ml). The method of analysis used was similar to that described by TAYLOR *et al.* (19). The S^{35} determination is considered a direct measurement of the unchanged cyclamate (19). In some experiments, cold sodium or calcium cyclamate was used and analyzed by the gravimetric method described by RICHARDS *et al.* (3). Sodium sulfate-S^{35} was used in conjunction with anhydrous sodium sulfate (2.6 to 12.7 × 10^6 counts/minute/ml). The procedure of analysis was similar to that adopted for the cyclamate-S^{35}. A very small amount of the sulfate may be retained in the body or excreted in the urine as ethereal sulfates (18). This was not determined in the present experiments. The various experimental procedures used will be described separately as they are presented.

I. *Absorption, excretion and retention of sodium cyclamate, calcium cyclamate and sodium sulfate after oral ingestion of large amounts by various animals*

A. *Excretion of sodium cyclamate, calcium cyclamate and sodium sulfate by the rat after single oral doses.*

Twenty-two rats of both sexes were used in this experiment. All the rats with the exception of two which received calcium cyclamate were kept in separate individual metabolism cages. The urine and feces were collected separately by a technique similar to that described by FINK *et al.* (20). Two rats were placed in modified Bollman's holders (21) to assure freedom from the possible contamination of the urine when the stool became loose at the high dose.

Single doses of sodium cyclamate, calcium cyclamate or sodium sulfate-S^{35} (all in

TABLE I (Continued)

Excretion of Sodium Sulfate-S^{35}, Sodium Cyclamate and Calcium Cyclamate in the Urine and Feces of the Rat after Various Oral Doses

Wt. Gram	Sex	Treatment	Dose g/kg	Excretion, % of the Oral Dose										
				0 to 8 hrs		8 to 24 hrs		24 to 48 hrs		48 to 72 hrs		Total		
				Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces	Sum
225	f	Na cyclamate	0.50	18.3	31.6	8.9	37.8	7.0	4.5	1.4	0.0	35.6	73.9	109.5
265	m		0.50	21.4	(—)	9.0	60.3	0.0	0.6	0.0	0.0	30.4	—	—
			ave.	19.9		9.0	49.2	3.5	2.6	0.7	0.0	33.0		
220	f		1.00	14.8	12.5	7.9	48.9	1.3	0.5	0.0	0.0	24.0	61.9	85.9
265	m		1.00	14.4	6.7	6.4	34.7	0.3	1.8	0.0	36.0	2[illegible]	79.2	100.3
			ave.	14.6	9.6	7.2	41.8	0.8	1.2	0.0	18.0	22.6	70.6	93.1
240	f		2.00	18.6	12.7	11.3	24.6	0.6	14.6	0.6	8.8	31.1	60.7	91.8
287	m		2.00	12.3	37.0	16.2	32.8	3.9	8.2	1.3	2.5	33.7	80.5	114.2
			ave.	15.5	24.9	13.8	28.7	2.3	11.4	2.0	5.7	32.4	70.6	103.0

TABLE I (Continued)

Excretion of Sodium Sulfate-S^{35}, Sodium Cyclamate and Calcium Cyclamate in the Urine and Feces of the Rat after Various Oral Doses

Wt. Gram	Sex	Treatment	Dose g/kg	Excretion, % of the Oral Dose										
				0 to 8 hrs		8 to 24 hrs		24 to 48 hrs		48 to 72 hrs		Total		
				Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces	Sum
240	m	Ca cyclamate	0.46	13.5	1.7	8.5	80.9	0.4	0.5	0.0	0.0	22.4	83.1	105.5
220	f		0.89	9.3	0.4	11.0	55.2	0.7	17.8	3.9	3.1	24.9	76.5	101.4
170	f		0.93	9.3	(—)	4.5	46.6	0.8	20.4	0.0	2.5	14.6	—	—
190	f		0.99	7.6	.2	3.8	52.2	0.4	19.9	0.0	11.6	11.8	83.9	95.7
			ave.	8.7	0.3	6.4	51.3	0.6	19.4	1.3	5.7	17.1	80.2	98.6
185	f		1.85	12.9	5.6	17.6	54.2	0.6	3.9	0.0	2.3	31.1	66.0	97.1
205	f		1.85	20.1	41.1	5.9	20.9	0.8	5.6	1.3	2.8	28.1	70.4	98.5
245	f		1.85	15.1	5.3	11.7	43.6	2.6	9.9	0.0	6.4	29.4	65.2	94.6
245	f		1.85	15.4	39.8	12.5	10.0	1.9	18.0	1.3	8.2	31.1	76.0	107.1
			ave.	15.9	23.0	11.9	32.2	1.5	9.4	0.7	4.9	29.9	69.4	99.4

* Figure in parenthesis in this and the next table is the value for the mixed specimens. (—) No accurate values were obtained.

TABLE II. — *Fate of Sodium Cyclamate-S^{35} and Sodium Sulfate-S^{35} in the Rat at the End of 4 and 8 Hours after Various Single Doses*

Wt. Gram	Sex	Treatment	Dose g/kg	Total Dose mg	Exptl. Period Hours	Total Recovery (% of Total Dose)	% Distribution					
							Unabsorbed				Absorbed	
							Total	GI Content	Excreted: Feces	Excreted: Urine	Body	Total
202	f	Na Cyclamate	2.0	405	4	97.9	70.3	60.0	10.3	21.8	7.9	29.7
194	f	Na Cyclamate	2.0	388	4	101.6	79.0	44.2	34.8	16.5	4.5	21.0
						ave.	74.65	52.1	22.55	19.15	6.2	25.35
188	f	Na Cyclamate	2.0	375	8	89.5	80.6	62.5	18.1	16.0	3.4	19.4
182	f	Na Cyclamate	2.0	365	8	97.6	78.1	51.1	27.0	18.4	3.5	21.9
						ave.	79.35	56.8	22.55	17.2	3.45	20.65
190	f	Na Cyclamate	4.0	760	4	89.2	85.3	79.3	6.0	7.3	7.4	14.7
200	f	Na Cyclamate	4.0	800	4	103.3	85.3	79.1	6.2	5.1	9.6	14.7
						ave.	85.3	79.2	6.1	6.2	8.5	14.7
202	f	Na Cyclamate	4.0	810	8	94.2	79.0	63.4	15.6	15.5	5.5	21.0
192	f	Na Cyclamate	4.0	767	8	100		17.5	(79.9)		2.6	
						ave.		40.45			4.05	

Table II (Continued). — *Fate of Sodium Cyclamate-S^{35} and Sodium Sulfate-S^{35} in the Rat at the End of 4 and 8 Hours after Various Single Doses*

Wt. Gram	Sex	Treatment	Dose g/kg	Total Dose mg	Exptl. Period Hours	Total Recovery (% of Total Dose)	% Distribution					
							Unabsorbed				Absorbed	
							Total	GI Content	Excreted: Feces	Excreted: Urine	Body	Total
195	f	Na_2SO_4	1.4	273	4	88.4	49.4	37.2	12.2	41.3	9.3	50.6
195	f	Na_2SO_4	1.4	273	4	91.6	63.3	46.2	17.1	30.9	5.8	36.7
						ave.	56.35	41.7	14.65	36.1	7.55	43.65
210	f	Na_2SO_4	1.4	294	8	88.0	52.5	37.8	14.4	41.0	6.8	47.8
199	f	Na_2SO_4	1.4	278	8	99.5	59.0	15.2	43.8	36.7	4.3	41.0
						ave.	55.6	26.5	29.1	38.85	5.55	44.4
164	f	Na_2SO_4	2.8	459	4	83.4	66.2	54.7	11.5	21.9	11.9	33.8
220	f	Na_2SO_4	2.8	616	4	95.7	74.6	61.4	13.2	18.3	7.1	25.4
						ave.	70.4	58.05	12.35	20.1	9.5	29.6
194	f	Na_2SO_4	2.8	507	8	108.4	60.2	14.9	45.3	37.0	2.8	39.8
202	f	Na_2SO_4	2.8	564	8	94.6		15.1	(75.5)		9.4	
						ave.		15.0			6.1	

TABLE III

Urinary Excretion and Blood Level of Sodium Cyclamate-S^{35} After Oral Doses in the Dog

Dog (Wt, kg and Sex)	Dose g/kg	Observation Period (hours)	Condition of feces, if any	Urinary Excretion Of S^{35}; % of Oral Dose	Blood Concentration of Na Cyclamate-S^{35} μg/ml
R (12.5, m)	0.75	11	Loose (1)	16.6	26
		17.5		20.7	
F (12.7, f)	1.0	17		64.9	
B (9.8 f)	1.0	17		26.7	

(1) Analysis of this sample gives the following results: Water: 84.1 %; sodium cyclamate: 21.4 mg/g when wet, 134.3 mg/g in dried solid, 25.4 mg/ml in the free liquid.

group of rats (used in a separate experiment described in detail in Section V), blood was taken from the heart one and one-half hours after various oral doses. This particular group of rats was given water but no food for 48 hours prior to the experiment. The water was withdrawn two hours before the oral dose and nothing allowed during the experiment.

The results are summarized in Table IV. These show that over a wide range of equimolar doses and time periods, the blood concentrations of sodium sulfate were always much higher than those of sodium cyclamate. This appears to be readily explained by the higher rate of absorption of sodium sulfate. The only exception was seen at the high dose (20 mM/kg) at the eighth hour. At this dose the blood concentration of the sulfate appeared to decline rapidly toward the eighth hour while that of the cyclamate remained little changed. However, at the lower doses, both sulfate and cyclamate concentrations in the blood tend to decrease with time. The observed individual variations do not permit additional inference.

II. *Determination of the apparent degree of ionization of sodium sulfate, sodium cyclamate and calcium cyclamate*

If the laxative action of the cyclamates is due to the same mechanism as sodium sulfate it appears important to compare their osmotic pressures in solutions at the same concentrations which were found in the alimentary tract after oral laxative doses.

The apparent degree of ionization was determined for each salt at the concentrations which are theoretically isosmotic with 0.9 % NaCl if completely ionized. Two additional concentrations of sodium cyclamate were also chosen to cover a wider range of experimental conditions. Values for the freezing point depression of these solutions were determined in duplicate by means of a Beckman freezing point apparatus. The apparent

TABLE IV

Blood Concentration of Sodium Sulfate-S^{35} and Sodium Cyclamate-S^{35} in the Rat

Wt. Gram	Sex	Treatment Sodium Sulfate g/kg (mM/kg)	Time, Hours	Blood Concentration μg/ml	Wt Gram	Sex	Treatment Sodium Cyclamate g/kg (mM/kg)	Time, Hours	Blood Concentration μg/ml
255	m	0.7 (5)	1.5	175.0	117	f	1.0 (5)	1.5	86.7
135	f	0.7 (5)	1.5	188.7	132	f	1.0 (5)	1.5	78.6
118	f	0.7 (5)	1.5	165.4					
302	m	1.4 (10)	1.5	167.6	259	m	2.0 (10)	1.5	70.2
141	f	1.4 (10)	1.5	192.3	137	f	2.0 (10)	1.5	108.4
129	f	1.4 (10)	1.5	214.5	124	f	2.0 (10)	1.5	88.4
195	f	1.4 (10)	4.0	103.6	202	f	2.0 (10)	4.0	51.4
195	f	1.4 (10)	4.0	483.6	194	f	2.0 (10)	4.0	26.9
210	f	1.4 (10)	8.0	52.2	188	f	2.0 (10)	8.0	25.9
199	f	1.4 (10)	8.0	341.2	182	f	2.0 (10)	8.0	18.6
164	f	2.8 (20)	4.0	164.0	200	f	4.0 (20)	4.0	61.1
220	f	2.8 (20)	4.0	109.1					
194	f	2.8 (20)	8.0	38.1	202	f	4.0 (20)	8.0	83.9
202	f	2.8 (20)	8.0	33.0	192	f	4.0 (20)	8.0	68.1

degree of ionization of each solution was then calculated according to procedures prescribed by Van't Hoff and Arrhenius. The results are summarized in Table V. They show that while sodium cyclamate is completely ionized at a range of concentration from 0.1 to 0.2 M, calcium cyclamate at 0.1 M and sodium sulfate at 0.098 M are apparently ionized only 90 % and 65 %, respectively.

TABLE V

Determination of the Apparent Degree of Ionization

Compound (mol wt)	Concentration of solutions tested				Apparent degree of ionization
	M/liter	mg/ml	mOsm/liter if completely ionized to independent ions	mOsm/liter from freezing point data	
Sodium sulfate (142.06)	0.098	14	300	230	65 %
Na cyclamate (201.33)	0.100	20	200	200	100 %
	0.150	30	300	300	100 %
	0.200	40	400	400	100 %
Ca cyclamate (432.58)	0.100	43	300	280	90 %

III. *The laxative effect of sodium cyclamate, calcium cyclamate and sodium sulfate in the rat, mouse and dog*

In accord with previous observations (1, 2) laxative effects were produced by single oral doses of either cyclamate or sodium sulfate in the rat, mouse or dog.

The criteria for a laxative effect were based upon the changes in the physical state of the fecal discharge. An arbitrary scoring system was adopted as follows to describe the physical condition of the feces.

I — Formed feces with definite shape.
III — Soft, more moisture, losing shape.
V — Loose, high water content, no recognizable shape.
VII — Watery or mucoid.

Intermediate conditions are scored as II, IV and VI.

According to these criteria, the feces of healthy rats and mice in the stock corresponded to scores I or II. Animals whose feces corresponded to a score of III were eliminated from the experiment.

Dogs confined in cages showed a marked variation in the physical condition of the feces from day to day. Some dogs had stools scored as I or II once in a few days, while others had soft stools (III or IV) every day.

A. *Rat experiments.*

All rats were individually housed in cages with wide-mesh (2/in) bottoms. Food and water were allowed ad lib. before and during the experiment. Clean cardboard was placed a few inches under the cage. Feces accumulated on the board could be easily examined for scoring. Water solutions of sodium cyclamate, calcium cyclamate and sodium sulfate were given orally to the rat in appropriate concentrations so that the different dosages were always given in a constant volume of 40 ml/kg. Each solution contained 5 % carbon (Norite) suspension for the purpose of measuring the gastrointestinal motility which will be described in Section IVC.

Four experiments were performed with 36 rats. Each experiment was separated from the other by at least one week. Eight or nine rats including equal or about equal number of both sexes were tested at each dose level in each experiment. Three or four rats of both sexes receiving 40 ml of water (containing 5 % carbon suspension) per kg of body weight were used as controls in each experiment. The rats were kept under observation for periods of 8 to 11 hours. The character of the fecal discharge was recorded hourly. The control rats never showed any abnormalities in their fecal formation during these experiments.

Among the treated rats marked variations were observed regarding the onset, duration and severity of the response to the single laxative dose of any one of these three compounds. The change of the character of the feces usually began from two to four hours after the dose. Occasionally it occurred as early as 1½ hours. Deviations of the fecal condition from the control seldom lasted beyond four hours in any of the groups. The effect was considered *a positive laxative response* when the feces changed from I or II to III or above within 8 hours following the dose.

Following the various oral doses of these compounds the percentage of animals showing a positive response in a period of 8 hours was calculated. The data and results are given in Table VI. Because marked individual and daily variations were observed, the data from all rats were treated as from different animals even when they were used repeatedly.

It should be noted in this table that the corrected doses are equivalent to the corresponding oral doses after the latter were corrected for absorption from the alimentary tract during the eight hours of the experiment and also for the apparent degree of ionization. The average values for the percentage of absorption during the period of 8 hours are taken from Table II. The unabsorbed portions of sodium cyclamate and sodium sulfate are 79.2 % and 57.1 % respectively. For the absorption of calcium cyclamate, an arbitrary value, 16.75 % is used, which is the sum of the average value of urinary excretion in 8 hours, i.e., 13 % (from TABLE I) plus the average cyclamate retention in the body, i.e., 3.75 % (from TABLE II). Accordingly a value of 83.25 % is taken for the unabsorbed portion of calcium cyclamate in 8 hours after oral doses. The values for the apparent degree of ionization were obtained from Table V. The oral doses in g/kg of body weight are thus

10 % w/v solutions) were given orally by a metal feeder. Urine and feces were collected for analysis at the end of 8, 24, 48 and 72 hours. The data are summarized in Table I. A large portion (about 70 to 90 %) of the various oral doses of all these three compounds is excreted by the rat in the first 24 hours. However, the cyclamate salts were largely unabsorbed and excreted in the feces while sodium sulfate was largely absorbed and excreted in urine. The percentages of ingested sulfate excreted in the urine and feces at the end of 24 hours are comparable at all dosage levels in both sexes. This also appears true for sodium cyclamate. Calcium cyclamate apparently is absorbed to a slightly lesser extent than sodium cyclamate at the lower dosage levels.

B. *Fate of sodium cyclamate-S^{35} and sodium sulfate-S^{35} in the rat.*

Sixteen female rats were used in this experiment. Each rat was confined to a modified Bollman's holder for separate collection of urine and feces. Sodium cyclamate-S^{35} at doses of 2.0 and 4.0 g/kg and sodium sulfate-S^{35} at doses of 1.4 and 2.8 g/kg were given orally and respectively to four groups of four rats each. Each dose was given in a final volume of 40 ml of water per kg. Food and water were allowed ad lib. before and during the experiment. At the end of 4 or 8 hours after each dose the urinary bladder and rectum were carefully emptied by pressure and the animals sacrificed by administering chloroform. The animals were then opened along the median line of the abdomen and symphysis pubis. Ligatures were placed at the lower end of the esophagus and just above the anus. The whole gastrointestinal tract was carefully removed. The contents of the entire tract were drained and then emptied by a gentle milking process into a tared beaker. The emptied gut was returned to the carcass which was then weighed and later dissolved in 25 % potassium hydroxide on the steam bath and homogenized after acidification with nitric acid. Analyses showed that about 89 to 100 % of original dose of sodium cyclamate-S^{35} and 83 to 100 % of the original dose of sodium sulfate-S^{35} were recovered. The data are shown in Table II with the percentage distribution of the total amount of each salt recovered from the gastrointestinal content, feces, urine and the carcass. Although there are certain individual variations, the unabsorbed percentages of the various doses of sodium cyclamate and sodium sulfate are generally comparable after a period of 4 or 8 hours. The fraction retained in the carcass was relatively small and decreased with time while the urine content apparently increased. It may be noted that percentagewise the excreted amounts of both compounds in the urine and feces at the end of 8 hours were comparable to the corresponding values shown in Table I.

C. *Excretion studies in dogs.*

Three dogs were separately confined in metabolism cages. Water was allowed ad lib. and the usual daily feeding was offered. Sodium cyclamate-S^{35} solution in water was given by stomach tube at doses of 0.75 g/10 ml/kg to one dog and 1.0 g/10 ml/kg to the other two. Urine was obtained by catheterization and pooled with the amount, if any, collected in the receptacle under the cage. Only one dog excreted feces during the observation period; this was a loose stool passed 11 hours after the dose. Part of the fresh specimen was collected and analyzed. The results are given in Table III, including the blood level in one dog. It can be seen that while the urinary excretion varied markedly among these three dogs, the amounts excreted by two of the dogs (R and B) are fairly close to the percentage values found in the rat (Table I).

D. *Blood concentrations of sodium cyclamate-S^{35} and sodium sulfate-S^{35} after oral administration in the rat.*

Blood for analysis was obtained by heart puncture from most of the rats before the end of the experiment (4 and 8 hours post-treatment) described in section IB. In another

Table I

Excretion of Sodium Sulfate-S^{35}, Sodium Cyclamate and Calcium Cyclamate in the Urine and Feces of the Rat after Various Oral Doses

Wt. Gram	Sex	Treatment	Dose g/kg	Excretion, % of the Oral Dose: 0 to 8 hrs Urine	0 to 8 hrs Feces	8 to 24 hrs Urine	8 to 24 hrs Feces	24 to 48 hrs Urine	24 to 48 hrs Feces	48 to 72 hrs Urine	48 to 72 hrs Feces	Total Urine	Total Feces	Total Sum
210	m	Na_2SO_4	0.35	48.1	10.8	11.9	6.9	5.0	2.0	(5.9)*	0.3	—	25.0	90.6
205	f		0.35	56.7	.2	9.5	22.3	1.9	2.2	0.5		68.6		93.6
			ave.	52.4	5.5	10.7	14.6	3.5	2.1					92.1
195	f		0.70	37.5	15.6	15.9	17.8	3.6	2.4	1.0	0.3	58.0	36.1	94.1
245	m		0.70	52.0	10.5	9.3	20.5	2.3	2.2	0.5	0.3	64.1	33.5	97.6
			ave.	44.8	13.1	12.6	19.2	3.0	2.3	0.8	0.3	61.1	34.8	95.9
225	f		1.41	45.8	14.8	7.3	14.0	2.7	2.7	1.1	0.5	56.9	32.0	88.7
340	m		1.41	38.9	8.8	20.7	14.1	2.3	2.7	1.0	0.7	62.9	26.3	89.2
			ave.	42.4	11.8	14.0	14.1	2.5	2.7	1.1	0.6	59.9	29.2	89.0
218	f		2.00	45.2	8.6	15.5	4.5	5.2	2.3	1.1	1.3	67.0	16.7	83.7
270	m		2.00	58.4	9.5	13.1	9.9	2.0	1.1	0.9	0.7	74.4	21.2	95.6
			ave.	51.8	9.1	14.3	7.2	3.6	1.7	1.0	1.0	70.7	19.0	89.7

10 % w/v solutions) were given orally by a metal feeder. Urine and feces were collected for analysis at the end of 8, 24, 48 and 72 hours. The data are summarized in Table I. A large portion (about 70 to 90 %) of the various oral doses of all these three compounds is excreted by the rat in the first 24 hours. However, the cyclamate salts were largely unabsorbed and excreted in the feces while sodium sulfate was largely absorbed and excreted in urine. The percentages of ingested sulfate excreted in the urine and feces at the end of 24 hours are comparable at all dosage levels in both sexes. This also appears true for sodium cyclamate. Calcium cyclamate apparently is absorbed to a slightly lesser extent than sodium cyclamate at the lower dosage levels.

B. *Fate of sodium cyclamate-S^{35} and sodium sulfate-S^{35} in the rat.*

Sixteen female rats were used in this experiment. Each rat was confined to a modified Bollman's holder for separate collection of urine and feces. Sodium cyclamate-S^{35} at doses of 2.0 and 4.0 g/kg and sodium sulfate-S^{35} at doses of 1.4 and 2.8 g/kg were given orally and respectively to four groups of four rats each. Each dose was given in a final volume of 40 ml of water per kg. Food and water were allowed ad lib. before and during the experiment. At the end of 4 or 8 hours after each dose the urinary bladder and rectum were carefully emptied by pressure and the animals sacrificed by administering chloroform. The animals were then opened along the median line of the abdomen and symphysis pubis. Ligatures were placed at the lower end of the esophagus and just above the anus. The whole gastrointestinal tract was carefully removed. The contents of the entire tract were drained and then emptied by a gentle milking process into a tared beaker. The emptied gut was returned to the carcass which was then weighed and later dissolved in 25 % potassium hydroxide on the steam bath and homogenized after acidification with nitric acid. Analyses showed that about 89 to 100 % of original dose of sodium cyclamate-S^{35} and 83 to 100 % of the original dose of sodium sulfate-S^{35} were recovered. The data are shown in Table II with the percentage distribution of the total amount of each salt recovered from the gastrointestinal content, feces, urine and the carcass. Although there are certain individual variations, the unabsorbed percentages of the various doses of sodium cyclamate and sodium sulfate are generally comparable after a period of 4 or 8 hours. The fraction retained in the carcass was relatively small and decreased with time while the urine content apparently increased. It may be noted that percentagewise the excreted amounts of both compounds in the urine and feces at the end of 8 hours were comparable to the corresponding values shown in Table I.

C. *Excretion studies in dogs.*

Three dogs were separately confined in metabolism cages. Water was allowed ad lib. and the usual daily feeding was offered. Sodium cyclamate-S^{35} solution in water was given by stomach tube at doses of 0.75 g/10 ml/kg to one dog and 1.0 g/10 ml/kg to the other two. Urine was obtained by catheterization and pooled with the amount, if any, collected in the receptacle under the cage. Only one dog excreted feces during the observation period; this was a loose stool passed 11 hours after the dose. Part of the fresh specimen was collected and analyzed. The results are given in Table III, including the blood level in one dog. It can be seen that while the urinary excretion varied markedly among these three dogs, the amounts excreted by two of the dogs (R and B) are fairly close to the percentage values found in the rat (Table I).

D. *Blood concentrations of sodium cyclamate-S^{35} and sodium sulfate-S^{35} after oral administration in the rat.*

Blood for analysis was obtained by heart puncture from most of the rats before the end of the experiment (4 and 8 hours post-treatment) described in section IB. In another

Table I

Excretion of Sodium Sulfate-S^{35}, Sodium Cyclamate and Calcium Cyclamate in the Urine and Feces of the Rat after Various Oral Doses

Wt. Gram	Sex	Treatment	Dose g/kg	Excretion, % of the Oral Dose											
				0 to 8 hrs		8 to 24 hrs		24 to 48 hrs		48 to 72 hrs		Total			
				Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces	Sum
210	m	Na_2SO_4	0.35	48.1	10.8	11.9	6.9	5.0	2.0	(5.9)*	0.3	—	25.0	90.6
205	f		0.35	56.7	.2	9.5	22.3	1.9	2.2	0.5		68.6		93.6
			ave.	52.4	5.5	10.7	14.6	3.5	2.1		0.3	58.0	36.1	92.1
195	f		0.70	37.5	15.6	15.9	17.8	3.6	2.4	1.0	0.3	64.1	33.5	94.1
245	m		0.70	52.0	10.5	9.3	20.5	2.3	2.2	0.5				97.6
			ave.	44.8	13.1	12.6	19.2	3.0	2.3	0.8	0.3	61.1	34.8	95.9
225	f		1.41	45.8	14.8	7.3	14.0	2.7	2.7	1.1	0.5	56.9	32.0	88.7
340	m		1.41	38.9	8.8	20.7	14.1	2.3	2.7	1.0	0.7	62.9	26.3	89.2
			ave.	42.4	11.8	14.0	14.1	2.5	2.7	1.1	0.6	59.9	29.2	89.0
218	f		2.00	45.2	8.6	15.5	4.5	5.2	2.3	1.1	1.3	67.0	16.7	83.7
270	m		2.00	58.4	9.5	13.1	9.9	2.0	1.1	0.9	0.7	74.4	21.2	95.6
			ave.	51.8	9.1	14.3	7.2	3.6	1.7	1.0	1.0	70.7	19.0	89.7

TABLE VI

The Laxative Response and the Gastrointestinal Motility of Rats after Various Oral Doses of Sodium Cyclamate, Calcium Cyclamate, and Sodium Sulfate

No. of rats	Wt. range g	Oral dose g/40 ml/kg	Corrected dose* mOsm/kg	Positive laxative response % in 8 hours	Animals passing C in stools % in 8 hours**
Water					
3	109–126			0.0	0.0
4	172–271			0.0	0.0
4	172–281			0.0	0.0
4	182–291			0.0	0.0
Na cyclamate					
8	88–124	1.50	11.80	50.0	87.5
8	191–319	1.50	11.80	0.0	37.5
8	184–280	1.71	13.45	12.5	62.5
8	203–312	2.00	15.74	50.0	75.0
8	107–134	2.00	15.74	62.5	87.5
8	178–268	3.42	26.91	100.0	100.0
Ca cyclamate					
8	190–288	1.30	7.01	0.0	0.0
8	188–292	2.60	14.01	37.5	100.0
8	192–294	3.68	19.83	75.0	87.5
8	191–292	5.20	28.02	100.0	100.0
Na_2SO_4					
8	178–268	1.22	11.28	0.0	50.0
8	94–137	1.40	12.95	25.0	37.5
8	200–309	1.40	12.95	50.0	62.5
8	183–268	1.63	15.08	62.5	75.0
8	198–285	2.80	25.90	100.0	87.5
9	104–123	2.80	25.90	89.0	88.9

* This is converted from the corresponding oral dose after corrections for the factors of absorption and the apparent degree of ionization. The calculations for the corrections are illustrated in Table VII.

** This measurement of gastrointestinal motility is described in the text (Section IVC) and Table XIII.

converted to the unabsorbed osmotic doses (as corrected) in milliosmols (mOsm) kg of body weight for each compound as shown by the calculations presented in Table VII. The term "milliosmol" used for the dosages indicates the amount of solute particles that would give rise to the same milliosmolar value when dissolved in one liter.

TABLE VII

The Conversion of Oral Dose in g/kg to the Corresponding Corrected or the Unabsorbed Osmotic Dose in milliosmols/kg for sodium cyclamate, calcium cyclamate and sodium sulfate

Compound	Oral Dose g/kg	Corrected dose mOsm/kg
Na cyclamate	1	$0.792^{*} \times \frac{2}{201.23} \times 1000$
Ca cyclamate	1	$0.8325^{*} \times \frac{1 + 2 \times 0.90}{432.58} \times 1000$
Na_2SO_4	1	$0.571^{*} \times \frac{1 + 2 \times 0.65}{142.06} \times 1000$

* The portion of the salt that is not absorbed by the rat in 8 hours.

TABLE VIII

Statistical Analysis of the Osmotic Dose-Laxative Response Data Shown in Table VI*

Treatment (No. of Rats)	ED_{50} Oral, mM/kg (g/kg)	ED_{50} Corrected, mOsm/kg** (95 % c.l.)	Slope Function** (95 % c.l.)
Na Cyclamate (48)	9.54 (1.92)	15.10 (13.3, 17.2)	1.37 (1.15, 1.63)
Ca Cyclamate (24)***	6.54 (2.83)	15.25 (12.3, 19.0)	1.37 (1.04, 1.81)
Na_2SO_4 (49)	11.27 (1.6)	14.8 (13.0, 16.8)	1.30 (1.14, 1.49)

* By the graphic method of Litchfield and Wilcoxon (23).

** No significant difference between potencies (corrected ED_{50}'s) and slope functions between any two groups.

*** The dose and effect values for the group receiving the lowest oral dose were deleted from the data because the expected effect was less than 0.01 for that dose.

Following the method of LITCHFIELD and WILCOXON (23) the corrected dose-per cent response data in Table VI were examined. The results of the analysis are shown in Table VIII. It can be seen that the ED_{50}, i.e., the corrected dose that produced a positive laxative response in 50 % of the animals during a period of 8 hours for each salt, and the slope function for each line have been estimated with their 95 % confidence limits. These lines are shown to be parallel to each other since there is no significant difference between the slope functions. The potency of each salt is not significantly different from the other when compared on the basis of the corrected ED_{50}.

It can be seen from the foregoing that although the oral doses in g or in mM per kg which produced a laxative effect in 50 % of the rats in 8 hours appear different for sodium cyclamate, calcium cyclamate and sodium sulfate, the corrected doses for these salts in mOsm per kg for the same laxative effect are practically identical. The findings seem to have an important bearing in the elucidation of the basic mode of action of these salts. If the cathartic action of sodium sulfate is due solely to its osmotic action in the alimentary canal, its potency can be accurately estimated only on the basis of the corrected ED_{50} as defined in the present study. The nearly equal potency of sodium cyclamate, calcium cyclamate, and sodium sulfate on this basis strongly suggests that the mechanism of action of their laxative effect is the same.

B. *Mouse experiments.*

Four experiments were carried out in 215 mice to study the laxative effect of sodium cyclamate, calcium cyclamate and sodium sulfate. The same procedures as described for the rat experiments were used except that each mouse was used only once for a period of 5 hours after the oral doses. During this period food and water were withheld and a change of stool from the normal score of I or II to III or above was considered as a positive response.

The results in the mouse were more variable than those in the rat. The mouse was also slightly more susceptible than the rat to these three compounds in the development of a positive response on the basis of the effective uncorrected oral doses. By the same method of analysis used in the rat experiments, the ED_{50} (g kg) and the 95 % confidence limits are as follows: sodium cyclamate, 1.30 (.87, 1.95); calcium cyclamate, 2.17 (1.53, 3.08); sodium sulfate, 0.74 (0.54, 1.02). In the absence of knowledge of the absorption rate of these compounds in the mouse under such experimental conditions the laxative effects could not be evaluated in terms of the corrected or unabsorbed osmotic doses as defined in the rat experiments just described. However, it is

interesting to note that the following figures are obtained when the oral ED_{50} (g/kg) is directly transformed into osmotic doses in terms of mOsm/kg body weight without consideration of the factor of absorption: sodium cyclamate, 12.9 (8.7, 19.4); calcium cyclamate, 14.0 (9.8, 19.9) and sodium sulfate, 12.0 (8.6, 16.8). These figures suggest that the rates of absorption of these salts in the mouse are comparable if they are assumed to be equally potent.

C. *Dog experiments.*

Experiments were performed in four dogs kept in single cages. Before each experiment the dogs were observed daily for a control period of at least five days to establish the frequency and consistency of the fresh fecal discharge. Only a clear-cut deviation of the score of the feces from the control condition following administration of each salt was taken as a positive response. Water solutions of sodium cyclamate or sodium sulfate were given by stomach tube to the dogs after 12 to 18 hours of fasting. Water ad lib. was allowed all the time and food was allowed after administration of the salt.

Positive responses usually occurred within 10 to 22 hours. The data are summarized in Table IX. The results indicate that a laxative effect may follow single oral administration of sodium cyclamate or sodium

TABLE IX

The Laxative Response of the Dog to Oral Doses of Sodium Cyclamate and Sodium Sulfate

Dog, Sex and Approx. Wt, kg	Treatment						
	Na Cyclamate g/kg*				Na_2SO_4 g/kg*		
	0.50	0.75	1.0	2.0	0.50	0.70	1.40
R, m, 12.5	o(3.5)	+(10)	+(5.6)				o(10)E
B, f, 9.8		o(5.0)	o(10)	+(10)E		+(10)	
F, f, 12.7		o(4.8)	o(10)	+(10)		o(10)	
W, f, 15	±(3.4)		+(5.5)		+(10)		
			±(10)				

* Different concentrations were used. The figures in parentheses are the volumes (ml) per kg at the various doses.
o No response.
± Doubtful response.
+ Positive laxation.
E Emesis occurred shortly after the dose.

sulfate at respective doses of 0.75 to 1.0 g and 0.5 to 0.7 g/kg body weight. Since the variation of the response was marked and the number of tests limited, the relative potencies of these two compounds in this species were not calculated.

IV. *Effect of sodium cyclamate, calcium cyclamate, sodium sulfate and other salts on the motility of the alimentary tract*

In order to investigate the possible effect of the cyclamates and sodium sulfate on the motility of the alimentary tract the following studies were made.

A. *Studies on the isolated intestine.*

Appropriate segments from the distal portion of the ileum and the proximal portion of the colon were isolated from 14 rats, 7 rabbits and 4 monkeys. In the colon preparation of the monkey only the taenia coli was used. All intestinal segments were suspended in constantly aerated baths (20 ml size) of Tyrode solution with temperature maintained at 37° C. All the solutions and transferring syringes were prewarmed to the same temperature. The solutions were added to the bath after withdrawing an equivalent amount of the Tyrode. The solutions used were approximately isotonic with the Tyrode after addition to the bath. Thus, aqueous solutions of the following concentrations (w/v) were used: sodium cyclamate, 3.0 %; calcium cyclamate, 4.32 %; sodium sulfate, 1.4 %; sodium chloride, 0.9 %; calcium chloride, 1.3 %; and calcium gluconate, 4.3 %.

In general, the results indicate that neither cyclamate nor sodium sulfate had any effect on the isolated intestine unless the salt concentration in the muscle bath far exceeded that found in the plasma of the rat as shown in Table IV. The calcium salts may either stimulate or depress the isolated gut, but such effects were all attributable to the calcium ions. It was shown in the laxative experiments in the rat that calcium cyclamate does not differ significantly from sodium cyclamate in potency and slope function. Thus the possible effects of the calcium ions on the intestine does not appear involved in the laxative response of the intact rat. Additional examination of the possible role of calcium ions *in vivo* will be made in later experiments.

B. *Studies in the mouse.*

a. Effect of parenteral administration of cyclamate salts.

The effect of intravenous injection of sodium cyclamate and calcium cyclamate on the motility of the alimentary canal was studied in 54 mice by a modified Macht technique in two series of experiments. A 5 % carbon suspension was given orally to each mouse in a volume of 10 ml/kg immediately following the intravenous dose. The various doses of the cyclamates and the saline for the controls were given in a volume of 40 ml/kg.

The mice were placed individually in small compartments with wide mesh (4/in) bottoms without food or water. The fecal discharge was scored every hour for 6 to 11 hours and the exact time for the first appearance of carbon in the stool was recorded.

The results were subjected to variance analysis after all the values for total passage time of the carbon were ranked and scored according to FISHER and YATES (24). No significant differences of the propulsive activities of the alimentary tract were found among groups receiving intravenous sodium cyclamate (30, 120, 500 and 1,000 mg/kg), calcium cyclamate (30 and 120 mg/kg) and the respective controls. None of the mice showed any deviation from the normal fecal consistency. These findings strongly suggest the lack of a significant influence of the absorbed portions of sodium or calcium cyclamate on the laxative response after oral doses.

b. Effect of oral administration of cyclamate and other salts.

Solutions of sodium cyclamate, calcium cyclamate, sodium sulfate and sodium chloride in various concentrations with 5 % carbon suspension were given orally to mice as a single dose in a volume of 40 ml/kg. A 5 % carbon suspension in water, 40 ml/kg, was used as control reference. The mice were sacrificed with chloroform at the end of 10 minutes and the percentage of the distance traversed by the carbon through the small intestine was measured. Marked individual variations occurred. In some of the mice the carbon appeared in the cecum at the end of 10 minutes. Although a period of 10 minutes was relatively short time, there were indications that some osmotic equilibrium had taken place. Following the doses of the cyclamate and sulfate salts, the intestine appeared to contain more fluid than the controls despite the fact that comparable volumes were given. However, actual measurements of the intestinal contents were not made.

The data and results are summarized in Table X. The doses in g/kg are represented in the osmotic form without correction for absorption. The absorption factor was not determined in this species, so that no attempt was made to evaluate the relative potencies of the salts. However, the findings suggest that there is an increase of motility with the increase of the osmotic dose. This relationship may be represented by the following equation: $\hat{Y} = 1.83\,X + 55.2$ ($\hat{Y}$ = % traversed of small intestine; X = mOsm/kg). The coefficient of correlation between the osmotic dose and the response of motility was found to be 0.687 which is highly significant ($P < 0.01$). Thus the results suggest that as the osmotic dose increases there is an increase of gastrointestinal motility and that this is probably a net result of the increase of bulk due to the osmotic retention of fluid.

C. *Studies in the rat.*

The gastrointestinal motility of the rat was examined by a modified Macht technique during the studies of the laxative actions of sodium cyclamate, calcium cyclamate and

TABLE X

Effect of Oral Doses of Sodium Cyclamate, Calcium Cyclamate, Sodium Sulfate and Sodium Chloride on the Gastrointestinal Motility of the Mouse

No. of Mice	Treatment (40 mg/kg Oral)	Dose		Ave. % Traverse of the small intestine, in 10 min ± s.e.
		g/kg	mOsm/kg	
29	Water		0.0	53.51 ± 2.51
8	NaCl	0.19	6.5	67.52 ± 3.90
19	Na Cyclamate	1.0	10.0	73.46* ± 3.11
14	Na Cyclamate	2.0	20.0	85.54* ± 11.93
10	Ca Cyclamate	0.98	6.3	62.37 ± 2.12
20	Na_2SO_4	0.7	11.3	85.14** ± 2.79

* One mouse of each group had a value of 100 %.
** Two mice of this group had a value of 100 %.

sodium sulfate, which were described in Section III-A. A 5 % carbon suspension was included in the solutions given orally to the rats. The time of the appearance of carbon in the stool was recorded for each rat. The percentage values for the different groups of animals that showed carbon in the stool at the end of 8 hours after treatment are given in Table VII.

It can be seen in general that the positive laxative response (as defined in Section III-A) is associated with a marked increase of the percentage of the animals showing carbon in the stool, which indicates increased propulsive motility of the gastrointestinal tract. The higher percentage values for increased motility over the corresponding laxative effects strongly suggest that some increase of gastrointestinal motility occurs at sublaxative doses.

The different groups were compared statistically by the method of LITCHFIELD and WILCOXON (21). The analysis and comparisons of these three compounds are shown in Table XI. No differences were found in potency (or ED_{50}, the dose promoting increased gastrointestinal motility in 50 % of animals at 8 hours) and slope function for the three compounds.

D. *Studies in the dog.*

Nineteen experiments were performed in 8 dogs which had undergone surgery to create various types of fistula or pouch preparations of the stomach or intestines as shown in Table XIV. Following recovery (at least two weeks) from the surgery all animals were trained to lie quietly on soft-padded boards during the period of observation. Pentobarbital anesthesia was administered to some of the animals which were excitable or had marked spontaneous activities of the gut. The motility of the gastric pouch or

TABLE XI

Statistical Analysis of the Osmotic Dose-Motility Response Data Obtained in the Rat as Shown in Table VI*

Treatment (no. of rats)	ED_{50}		Slope function** (95 % c.l.)
	Oral, mM/kg (g/kg)	Corrected, mOsm/kg** (95 % c.l.)	
Na cyclamate (48)	6.83 (1.37)	10.8 (8.92, 13.08)	1.55 (1.19, 2.02)
Ca cyclamate (32)	3.90 (1.73)	9.3 (6.15, 14.06)	1.82 (1.05, 3.15)
Na_2SO_4 (49)	9.90 (1.41)	13.0 (9.83, 17.19)	1.77 (0.93, 3.37)

* By the graphic method of Litchfield and Wilcoxon (23).
** No significant difference between the potencies (corrected ED_{50}'s) and slope functions between any two groups.

intestinal segment was recorded on a kymograph by means of a ballon-water manometer system. The balloon to be placed in the gut was made of a thin rubber condom, 5 cm in length, which could accommodate up to 25 ml of water without change of the pressure inside. It was mounted on the multi-eyed end of a urethral catheter (size 14F) to connect with the lower end of a water reservoir. The air-tight upper end of the reservoir was connected with a water manometer to monitor the intraluminal pressure changes (motility) of the gut. A control tracing for variable periods of time was always recorded before any test solution was given. Most of the intravenous doses were given in 1 ml/kg over a period of one minute.

The effects of variously administered doses and concentrations of sodium and calcium cyclamate on the motility are summarized in Table XII. Sodium cyclamate had no effect on any of the preparations in the 14 experiments which were made with intravenous doses ranging from 30 to 120 mg/kg. Intravenous administration of calcium cyclamate occasionally stimulated motility of the various segments (expts. 3, 8, 9, 10, 12 and 15). However, this effect of calcium cyclamate is explained by the presence of calcium, since calcium gluconate produced similar changes (expts. 3 and 10) whereas sodium cyclamate had no effect. It is of further interest that the stimulation was prevented by tetraethylammonium chloride (TEA), (expts. 3 and 10) suggesting that the action of the calcium was at the ganglionic or higher level. Rather high doses (often 60 mg/kg) of the calcium compounds were required to show

TABLE XII

Effect of Sodium and Calcium Cyclamates on the Motility of Various Fistula and Pouch Preparations in the Dog

Dog	Preparation	Expt. no.	I.V. administration (mg/kg)		Local instillation (vol., conc.)	
			Na cyclamate	Ca cyclamate	Na cyclamate	Ca cyclamate
Sh.*	Thiry fistula of proximal colon	1	± (15, 66, 75, 184)			
		9	P, o (30, 120)	P, o (30), + (60)		
		14			o (5 ml, 3 %)	o (5 ml, 3 %)
		16			+ (5 ml, 6 & 12 %) (1) o (2 ml, 3 & 12 %)	+ (5 ml, 6 & 12 %) o (5 ml, 3 %)
		19			+ (5 ml, 3, 6 & 12 %) (1) o (5 ml, 3, 6 & 12 %)	
Wh.*	Thiry fistula of proximal colon	2	o (30, 120)	o (30), ± (120)		
		13			o (5 ml, 12 %)	
		17	P, o (120)	P, o (15, 30, 60)	P, o (5 ml, 12 %)	P, + (5 ml, 12 %) (2)
		18			o (5 ml, 3 %)	o (5 ml, 3, 6 & 12 %) + (5 ml, 12 %)
So.	Thiry-Vella fistula of ileum	3	o (30, 120)	+ (30, 120) (3)		
		10	o (30, 120)	+ (30) (3)	± (10 ml, 12 %)	
Da.	Thiry-Vella fistula of ileum	4	o (30, 120)	o (30, 120)		
		11	P, o (30, 120)	P, o (30, 120)		
Tal.	Thiry-Vella fistula of jejunum	5	o (30, 120)	o (30, 60)		
		12	o (30, 120)	o (30), + (60)	o (10 ml, 3 & 12 %) (4)	o (10 ml, 3 & 12 %)

TABLE XII (Continued)

Effect of Sodium and Calcium Cyclamates on the Motility of Various Fistula and Pouch Preparations in the Dog

Dog	Preparation	Expt. no.	I.V. administration (mg/kg)		Local instillation (vol., conc.)	
			Na cyclamate	Ca cyclamate	Na cyclamate	Ca cyclamate
Bl.	Heidenhain pouch of fundus of stomach	6	o (30)	o (30), E (120)	o (10 ml, 3 & 12 %)	o (10 ml, 3 & 12 %)
		15	o (120)	+ (60) (7)		
Pa.	Pavlov pouch of fundus of stomach	7	o (30 (8), 60, 120 (8))	o (30, 60, 120)		
Re.	Heidenhain pouch of fundus of stomach	8	P, o (30, 60)	P, o (30), + (60)		

o No significant change of motility. + Increase of motility. ± Results inconclusive. E Emesis. P The observations were made in the dog under pentobarbital anesthesia. * Each dog also had a Thiry-Vella fistula of the ileum. Irrigation of this ileal loop with solutions of Na cyclamate (3 & 12 %) and Ca cyclamate (3 %) had no effect on the motility of the colon fistula (observations made during expts. 16 & 17).

(1) Instillation of 5.6 % Na_2SO_4 had no motor response. TEA abolished the activity and prevented the response to subsequent doses of sodium cyclamate.

(2) No effect was elicited by 5 ml of 1.4 % Na_2SO_4 but 5 ml of 0.9 % NaCl increased the activity.

(3) Abolished by TEA 10 mg/kg I.V.

(4) No more response after procaine (2 %, 5 ml) instillation to the fistula.

(5) Similar effect after calcium gluconate 30 mg/kg I.V. but not elicitable after TEA 10 mg/kg I.V.

(6) No effect was observed with 10 ml of Na_2SO_4 (1.4 % or 5.6 %).

(7) Subsequent dose of calcium gluconate 60 mg/kg caused emesis.

(8) Effect of carbaminoyl choline 1 μg/kg I.V. on the ileal fistula or the pouch was not changed after the I.V. doses of sodium cyclamate.

the stimulating effect. Emesis may follow subsequent or higher doses (expts. 6 and 15).

Local instillation of cyclamate solutions to the ileal or jejunal loop (expt. 12) or the Heidenhain pouch of the fundus of the stomach (expt. 15) produced no change of motility. A transient increase of activity of an ileal loop was observed in one dog (expt. 10) following instillation of 10 ml of sodium cyclamate solution (12 %). However, similar results were obtained with equal volumes of water or sodium sulfate solution (5.6 %). Apparently the transient increase of motility was not related to either cyclamate or sulfate. The colon fistula showed some interesting reactions to various solutions. In dog Sh., motor response was observed in two experiments (expts. 14 and 16) after instillation of sodium cyclamate solution but not after sodium sulfate of comparable concentrations. In the same experiments calcium cyclamate caused less consistent response of smaller magnitude than sodium cyclamate. Intravenous TEA (10 mg kg) abolished the motor response and prevented the response to subsequent treatment with sodium cyclamate. When attempts were made in another experiment to elucidate further the mechanism involved no response was obtained at any of the concentrations tested (expt. 19). The colon fistula of the other dog (Wh.) behaved differently. In contrast to no response after sodium cyclamate (expts. 13 and 17), a definite stimulating effect was observed with calcium cyclamate in two experiments (expts. 13 and 18) but not in another (expt. 17). The stimulating effect did not appear to be due to the hypertonicity of the solution since sodium cyclamate of comparable concentrations (12 %) also had no effect (expt. 13). However, the action of TEA (I.V.) and procaine (local) as shown in the table (expts. 13 and 18) suggests a local reflex action involving the ganglia.

The positive findings in these experiments are probably of no practical significance since they were not consistent and occurred usually with high concentrations of the test agents. It should also be noted that the concentration of sodium cyclamate in the free fluid of the loose stool of a dog with positive laxative response was approximately 2.5 % (see footnote in TABLE III). Also noteworthy is the fact that perfusion of sodium or calcium cyclamate solutions through the ileum caused no reflex changes of the colon in two dogs (see footnote in TABLE XII).

V. *Relation between the quantity of fluid retained and the osmotic pressure of sodium cyclamate and sodium sulfate in the gastrointestinal tract of the rat.*

Analysis of a number of observations in the rat described in the fore-

going sections has shown that the incidence of the laxative effect and the increase of gastrointestinal motility after oral doses of sodium cyclamate, calcium cyclamate and sodium sulfate are related to the osmotic activity of the unabsorbed solutes in the gastrointestinal tract. It should be noted that a significant correlation was obtained in both types of experiments in which the osmotic activity of the unabsorbed solutes was calculated from data of independent studies.

Although sodium sulfate is generally considered to cause a laxative effect by its osmotic retention of fluid in the gastrointestinal lumen, it appears necessary to secure direct evidence of its osmotic pressure and the volume of fluid retained in the unanesthetized intact animals. Since the cyclamate salts and sodium sulfate were found to be equally potent in producing laxative effect and an increase of gastrointestinal motility in the rat on the basis of the osmotic activity of the unabsorbed portion, these compounds should be expected to have a similar relationship between the osmotic pressure and the volume of fluid retention in the digestive tract if their mode of action is the same. This critical approach was carried out in the following experiments in the rat.

The following principles were adopted for these experiments. The digestive tract of the rat should contain as little solid material as possible at the time of the experiment. The dosages of the salts given should include doses that may give positive laxative response. Maximum time should be allowed for absorption and establishment of osmotic equilibrium. Finally, the total amount of intraluminal fluid in the digestive tract should be obtained for analysis before any is lost by the laxative effect.

In order to fulfill the requirements outlined above, experimental procedures described by CORI (25) were adopted with certain modifications. Rats in single cages with wide mesh (2/in) bottoms were fasted for 48 hours before and throughout the experiment. Water was allowed ad lib. until two hours before administering an equimolar oral dose of sodium cyclamate-S^{35} (2.0 and 1.0 g/40 ml/kg) or sodium sulfate-S^{35} (1.4 and 0.7 g/40 ml/kg). The time chosen for sacrificing the animals was 1 ½ hours after the dose because the previous experiments had shown that a laxative effect seldom occurs within this period of time. In all of the rats observed during the actual experiments, none discharged any fecal material during this period. However, a soft or liquid stool was found in the colon of most of the animals and in a few, this had nearly reached the rectum.

Twenty-four rats were used in five experiments. In each experiment, 4 rats were each given sodium sulfate-S^{35} or sodium cyclamate-S^{35} at different doses. The fifth rat in four of these experiments served as control for the amount of the free fluid in the gastrointestinal tract. The rats were sacrificed with chloroform; each segment of the gastrointestinal tract, namely stomach, small intestine, cecum and colon, was carefully tied off at the ends and junctions. Each segment was removed and its content emptied by a gentle milking process into a glass graduate. Usually very small amounts of solid were present except in the cecum. The free fluid was measured and analyzed for S^{35} after removing the residue by centrifuging.

The summarized data and the results of analysis of S^{35} and calculations are given in Table XIII. Inaccurate results obtained for one of the rats receiving sodium sulfate are excluded from the table.

TABLE XIII

The Volume of the Free Fluid and the Amount of the Unabsorbed Salt and its Concentration in the Gastrointestinal Tract of the Rat 1 1/2 hours after the Oral Doses of Sodium Cyclamate-S^{35} and Sodium Sulfate-S^{35}

Treatment 40 ml/kg oral	Rat wt. (g) & sex	Total dose mg	Unabsorbed dose			Fluid retained	
			% of total dose	g/kg	Corrected mOsm/kg	Total volume ml/100 g	Mean concentration mOsm/liter
Na cyclamate 5 % 2.0 g/kg	169 m	338	83.5	1.669	16.59	6.43	258.2
	259 m	520	66.9	1.344	13.36	4.75	281.4
	260 f	520	93.7	1.875	18.63	6.37	292.5
	137 f	274	64.6	1.293	12.85	4.90	262.3
	124 f	248	60.4	1.208	12.01	4.56	265.9
		ave.	73.83	1.478	14.688	5.40	272.06
		SE	6.330	0.126	1.254	0.411	6.443
Na cyclamate 2.5 % 1.0 g/kg	217 m	217	99.4	0.994	9.88	3.55	278.4
	272 m	272	59.7	0.597	5.93	2.74	216.6
	227 m	227	71.5	0.715	7.11	3.54	200.7
	117 f	117	79.7	0.797	7.92	3.40	232.7
	132 f	132	69.2	0.692	6.88	2.83	243.4
		ave.	75.90	0.759	7.544	3.21	234.36
		SE	6.68	0.066	0.664	0.177	13.177

TABLE XIII (Continued)

The Volume of the Free Fluid and the Amount of the Unabsorbed Salt and its Concentration in the Gastrointestinal Tract of the Rat 1 1/2 hours after the Oral Doses of Sodium Cyclamate-S^{35} and Sodium Sulfate-S^{35}

Treatment 40 ml/kg oral	Rat wt. (g) & sex	Total dose mg	Unabsorbed dose			Fluid retained	
			% of total dose	g/kg	Corrected mOsm/kg	Total volume ml/100 g	Mean concentration mOsm/liter
Na_2SO_4 3.5 % 1.4 g/kg	302 m	423.5	58.2	0.816	13.25	5.11	259.4
	266 f	372.3	62.3	0.872	14.35	6.21	231.1
	141 f	197.4	53.1	0.743	12.20	5.16	236.3
	129 f	180.6	54.4	0.762	12.33	4.61	267.5
		ave.	57.00	0.827	13.03	5.27	248.6
		SE	2.072	0.032	0.498	0.336	28.79
Na_2SO_4 1.75 % 0.7 g/kg	205 m	143.5	63.9	0.447	7.36	3.30	223.0
	255 m	178.5	54.1	0.379	6.29	2.99	210.2
	242 m	169.4	49.1	0.344	5.76	3.04	189.2
	135 f	94.5	24.2	0.170	2.86	1.65	173.5
	118 f	82.6	29.6	0.207	3.44	1.71	201.2
		ave.	44.18	0.309	5.14	2.54	199.4
		SE	7.494	0.053	0.858	0.355	8.52

The average volume of the free fluid in each segment of the gastrointestinal tract and its value in percentage of the total for each group of the rats 1½ hours after receiving different oral doses of sodium cyclamate or sodium sulfate are shown diagramatically with the standard errors in Figure 1. It shows a similar pattern of distribution between the two salts.

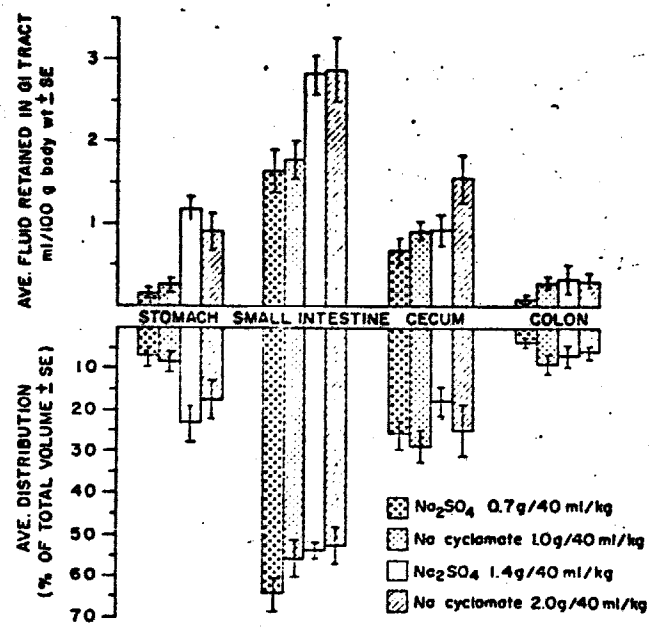

FIG. 1

Average volume and distribution of fluid retained in the gastrointestinal canal of the rat 1½ hours after various oral doses of sodium cyclamate-S^{35} and sodium sulfate-S^{35}.

The average osmotic concentration of the solute (corrected for the apparent degree of ionization calculated from the values in I.C.T. (22)) in the free fluid from the different digestive organs, and the distribution of the unabsorbed solutes in percentage of the total, 1½ hours after the treatment, are shown with their standard errors in Figure 2.

As previously summarized, certain relationships should exist between the mean osmotic activity of the total amount of the unabsorbed salt and the total amount of fluid retained. Accordingly, the relation between the volume (ml/100 g) of fluid retention and the calculated osmotic

dose (mOsm/kg) of the unabsorbed sodium sulfate and sodium cyclamate was critically examined. The respective values from each rat shown in Table XIII were plotted in Figure 3 where the equations for the two regression lines are also given.

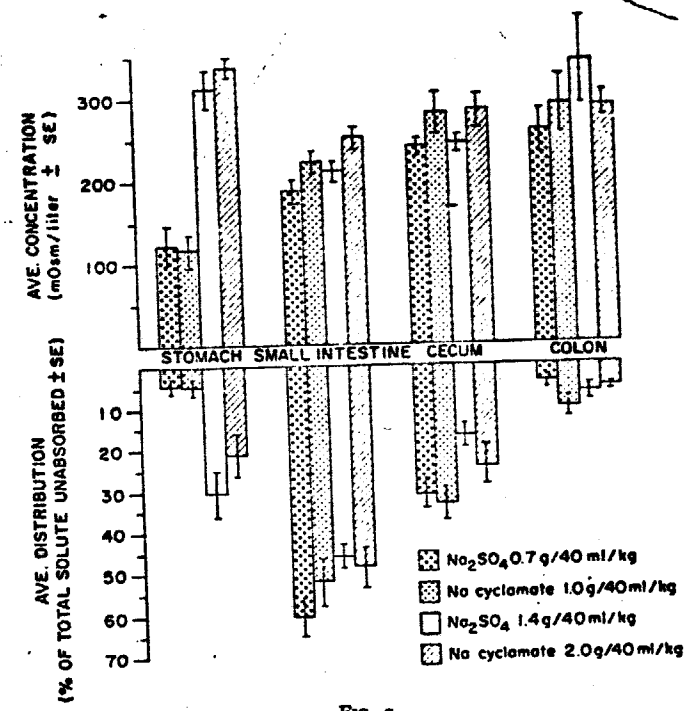

FIG. 2

Average concentration and distribution of sodium cyclamate-S^{35} and sodium sulfate-S^{35} in the gastrointestinal canal of the rat 1½ hours after various oral doses.

The coefficient of correlation (r) between the osmotic dose (the unabsorbed solute, mOsm/kg) for each salt and the effect of fluid retention (ml/100 g) was highly significant ($r = 0.985$ for sodium sulfate, 0.978 for sodium cyclamate). This relationship strongly supports the general concept regarding the mode of action of sodium sulfate as pointed out before. It also clearly demonstrates that the laxative action of sodium cyclamate is similar to that of sodium sulfate following oral doses.

Covariance analysis of the same data shows that there was no significant difference between either the slopes ($P \approx 0.075$) or the intercepts

(when X = O). The regression equation for the pooled data may be written as follows:

$$\hat{Y} = 0.320\,X + 0.864$$

where X is the dose of the unabsorbed salt (mOsm/kg) and $\hat{Y}$ is the estimated response of the amount of fluid retained (ml/100 g).

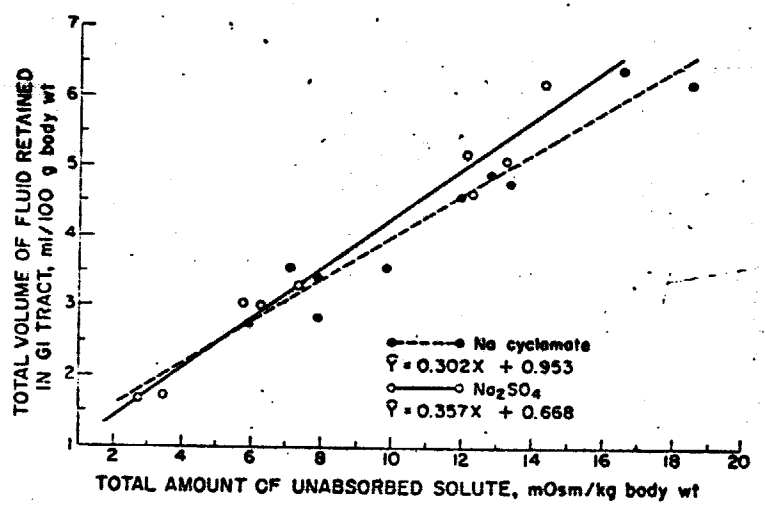

FIG. 3

Regression lines to show the relation between the volume (ml/100 g) of fluid retention and the corrected osmotic dose (mOsm/kg) of the unabsorbed sodium sulfate-S^{35} and sodium cyclamate-S^{35} 1½ hours after oral administration to the rat.

The lack of significant difference between the regression coefficients (slopes) and the intercepts for the sodium sulfate and sodium cyclamate data discussed above is interpreted as clear evidence that these two salts have a similar mode of action and comparable potency in the retention of fluid in the gastrointestinal tract of the rat. This is consistent with the previous observations in the rat that the potency of these salts as judged by the laxative and motor response is comparable on the basis of osmotic doses.

It is also interesting to note that in the control rats the measurable amount of the free gastrointestinal fluid had an average value of 0.82 ml/100 g (TABLE XIV) which does not differ significantly from the intercepts (when X = O) of the calculated regression lines discussed above.

TABLE XIV

Amount of Free Fluid in the Gastrointestinal Tract of the Control Rats After Removal of Food for 48 Hours and Water for 2 Hours

No. of Rats	Wt. grams	Sex	Amount of Fluid in GI Tract	
			Total Vol. ml	ml/100 grams
1	147	m	0.0	0.0
2	212	f	1.4	0.65
3	118	f	1.1	0.93
4	130	f	2.2	1.69
			ave.	0.82
			SE	0.22

It may be noted in Table XIII that all the mean concentrations (mOsm/liter) of the sodium cyclamate and sodium sulfate in the retained fluid were apparently hypotonic. However, the exact relationship between these partial osmotic pressures calculated for cyclamate or sulfate and the total osmotic pressure was not readily understood. Preliminary efforts were made to determine the total osmotic pressure by a micro-method of freezing point determination of the gastrointestinal fluid whenever the volume of the fluid permitted. The method was similar to that described by SIMONS (26). The results showed unexpected high values which were attributable to the bacterial activity as reported by FOLLANSBEE (27).

In order to examine more critically the same question regarding the partial versus the total osmotic pressure, several new experiments were conducted with 16 rats by the same procedures as described in this section. All gastrointestinal fluids were kept in tubes covered by Saran Wrap and immersed in ice water immediately after the collection. The freezing points of these specimens were determined by the same micro-method within two hours. Blood was taken from eight of these rats and defibrinated. The sera were kept cold for similar freezing point determinations. Several blood samples were collected and defibrinated under cover of mineral oil. The results were the same as those processed without the mineral oil. Sodium cyclamate-S^{35} and sodium sulfate-S^{35} were also determined in a large number of samples. The results are summarized in Table XV.

It can be seen that the total volume recovered and also the distribution of the volumes and solutes in the different segments were essentially in accord with the previous experiments (TABLE XIII, FIGURES 1 and 2). Since complete information of the solute was not available in most of the rats, the values obtained in these new experiments were not incorporated into the earlier data. However, the primary objective of these new experiments was achieved with respect to the total osmotic pressure of the gastrointestinal fluids and sera. (Many samples were deliberately examined repeatedly for their freezing points. Some samples showed an increase of osmotic pressure with time, particularly

The Volume and Osmotic Activity of the Free Fluid in the Gastrointestinal Tract and the Osmotic Activity of the Serum of the Rat 1 1/2 hours after the Oral Doses of Sodium Cyclamate-S^{35} and Sodium Sulfate-S^{35}

Treatment	Rat wt and sex	Stomach				Small intestine				Cecum			Colon				Serum mOsm/liter	Total	
		Vol ml	mOsm/liter Solute	mOsm/liter Total	Ratio S/T*	Vol ml	mOsm/liter Solute	mOsm/liter Total	Ratio S/T	mOsm/liter Solute	mOsm/liter Total	Ratio S/T	Vol ml	mOsm/liter Solute	mOsm/liter Total	Ratio S/T		Vol ml	ml/100 g
Na	159 f	1.65	290	368	.788	2.90	236	364	.6[illegible]00	256	375	.682	.25	209	—		321	7.80	4.90
cyclamate	171 f	.38		324		2.90	216	387	.5[illegible]35	282	331	.853	2.55	272	438	.621	327	8.68	5.06
5 %	170 f	.40		284		3.60	177	358	.4[illegible]10	257	350	.734	.65		326			9.75	5.74
2.0 g/kg	232 m	2.45		340		6.90		329	[illegible]20		339		.80		326			13.35	5.75
Na	160 f	.40		258		2.10	166	351	.4[illegible].85	219	372	.588	1.50	170	349	.487	302	4.85	3.03
cyclamate	168 f	.21		260		1.70	170	346	.4[illegible]2.30	194	342	.567	.33	113	354	.320	310	4.54	2.70
2.5 %	217 m	.60		334		5.30	193	329	.5[illegible]2.40	205	347	.590	.15					8.45	3.89
1.0 g/kg	228 m	1.05		279		4.90	218	316	.6[illegible]1.80	170	384	.442	0					7.75	3.40
Na_2SO_4	164 f	1.74	258	374	.690	4.20	238	332	.71[illegible]2.55	232	341	.679	2.20	230	335	.687	311	10.69	6.52
3.5 %	169 f	2.20	218	374	.580	3.70	212	348	.61[illegible]4.00	214	361	.592	.80	217	341	.636	310	10.70	6.33
1.4 g/kg	197 m	1.50				4.10		346	[illegible]4.90		349		1.70		338			12.20	6.19
	213 m	1.60				3.10		363	[illegible]5.68		352		.80		325			11.18	5.25
Na_2SO_4	165 f	.18		—		1.50	138	393	.35[illegible]2.05	231	305	.757	1.35	167	310	.541	307	5.08	3.08
1.75 %	182 f	.25		317		.90	128	329	.38[illegible]3.80	211	313	.673	.38	220	324	.679	303	5.33	2.93
0.7 g/kg	219 m	.10		362		1.40		372	[illegible]3.50	220	336	.654	.70		339			5.70	2.60
	234 m	.40		272		2.40	172	346	.49[illegible]3.70	179	362	.494	.20		353			6.70	2.86

* Ratio S/T denotes the ratio of the partial osmotic pressure contributed by the solute to the total osmotic pressure.

when kept at room temperature. These findings seem to support the opinion that the suppression of bacterial growth is important in the osmotic pressure determination (27)].

As shown in Table VII the calculated partial pressure of the solute (sodium sulfate or sodium cyclamate) of each specimen was relatively hypotonic to the sera whereas the total osmotic pressure was slightly hypertonic. Where both partial and total osmotic pressure values were obtained their ratio was calculated and shown in the same table. The overall means (± SE) were 0.59 (± .031) for the sodium cyclamate-treated rats and 0.60 (± .028) for the sodium sulfate-treated rats. These two mean values were practically identical.

VI. *Studies on the possible synergism between cyclamate and saccharin*

Saccharin is known to cause laxative effect in the animals following very large single oral doses (28). It therefore appeared interesting to examine whether the laxative effect of the cyclamate in the rat would be modified by simultaneous administration of saccharin. Various oral doses of calcium cyclamate were given. The effect was compared with that of calcium cyclamate combined with calcium saccharin. Since saccharin is about 10 times sweeter than cyclamate (29) the combined dose was administered in a ratio of 1 to 10. Accordingly, the total sweetness in 3.25 % calcium cyclamate with 0.325 % calcium saccharin was considered approximately equal to that in 6.5 % calcium cyclamate. The technique of the laxative study which has been described in Section III-A was used.

The general data and results are summarized in Table XVI. It can be seen that the addition of saccharin to the cyclamate at a 1 to 10 ratio caused laxation and hypermotility of the gastrointestinal tract comparable to those observed after the same single oral doses of the cyclamate.

Discussion

In accord with previous observations the changes in the stools after large oral doses of sodium and calcium cyclamates were confirmed in several species of experimental animals. Attempts were made to analyze the mechanism involved in close comparison with a known saline cathartic, sodium sulfate. This comparison requires a knowledge of the rate of absorption of each salt concerned under specific experimental conditions. Such information was thus acquired in intact rats with

Table XVI

The Laxative Response and Gut Motility Changes after Oral Doses of Calcium Cyclamate Alone or in Combination with Saccharin

Date	Treatment 40 ml/kg	Dose g/kg	No. of rats	Positive laxative response in 8 hours	Presence of C in stool in 8 hours (%)
11/17	Controls		4	0.0	0.0
12/8	Controls		4	0.0	0.0
11/17	Ca cyclamate 3.25 %	1.3	8	0.0	37.5
12/8	Ca cyclamate 3.25 %	1.3	8	0.0	25.0
12/8	Ca cyclamate 3.25 % with saccharin 0.325 %	1.3 + 0.13	8	12.5	25.0
11/17	Ca cyclamate 6.5 %	2.6	8	37.5	100.0
12/8	Ca cyclamate 6.5 %	2.6	8	100.0	100.0
12/8	Ca cyclamate 6.5 % with saccharin 0.65 %	2.6 + 0.26	8	62.5	75.0
11/17	Ca cyclamate 9.2 %	3.68	8	75.0	87.5
11/17	Ca cyclamate 12.95 %	5.2	8	100.0	100.0

wide range of doses including those resulting in laxation. It may be pointed out that whatever amount of the cyclamate or sodium sulfate is referred to in this report as the unabsorbed dose, this actually included the fraction that has been absorbed but re-excreted — a small fraction of the injected cyclamate (19) or sodium sulfate (30) is excreted through the bile; the sulfate may also pass into the upper small intestine from the blood or tissue fluid (30). It is noted that although the cathartic action of sodium sulfate has been known since the middle of the seventeenth century (31) and an active transportation mechanism has been recently demonstrated in the distal intestine (32, 33), there are no adequate quantitative absorption data for the evaluation of its cathartic action in intact animals.

Another factor generally overlooked in the consideration of the osmotic retention of fluid in the gastrointestinal tract by the unabsorbed salt is its apparent degree of ionization. In the present studies solutions of sodium and calcium cyclamate and sodium sulfate with certain selected concentrations were examined for their freezing points. Their apparent degree of ionization was then calculated (TABLE V).

The data on the apparent degree of ionization and the rate of absorption (in 8 hours) made possible the computation of the osmotic activity of the unabsorbed portions of the oral doses of each salt. The equipotent doses of these salts in producing the laxative effect and the increase in gut motility in the rat were thus found to be equi-osmotic in the gastrointestinal tract. These results suggest strongly that these three compounds have a similar mode of action and that the osmotic effect is highly probable as the common mechanism.

If the osmotic action of the unabsorbed salt is the sole basic mechanism of the laxative effect there should be no significant pharmacodynamic stimulation of the gastrointestinal tract by systemic action or local effect other than bulk retention. Extensive examination was carried out in the present studies in: (*a*) the isolated ileum and upper colon prepared from the rat, rabbit or monkey, (*b*) the mouse by intravenous doses and (*c*) the dog with various fistula or pouch preparations by intravenous or intraluminal doses. The results indicate no specific effect of cyclamate or sulfate ions in all these studies. It may be mentioned that similar conclusions regarding sodium sulfate were reached by DREYER and TSUNG (34) after intravenous doses in the cat. Increase of various degrees of motility of intestinal loop upon instillation or perfusion with sodium sulfate of various concentrations was reported (35, 36), but the bulk factor in the intestinal lumen was not entirely ruled out in these studies.

Additional evidence of the osmotic action of the unabsorbed salts was procured in the rat by direct examination of the correlation between the osmotic dose and volume of fluid retained. This proves to be highly significant and thus provides an important link for the interrelationship between the increase of gastrointestinal bulk, motility and the development of diarrhea with the osmotic activity of the unabsorbed salt. The assumption that the osmotic activity of sodium sulfate in the gut is the sole mechanism of action for its cathartic effect is firmly supported by the present series of experiments. It can also be concluded that the cyclamates are identical to sodium sulfate in the mode of action and specific potency in the production of laxation or catharsis.

It is of further interest that the calculated partial osmotic pressure of either the cyclamate or the sulfate (as sodium salts) in the gastrointestinal fluid averaged about 60 % of the total osmotic pressure. Therefore, the remainder must be contributed by other undetermined sources. This requires further exploration. It has been noted (11, 12, 14, 15) that the final osmotic pressure of the gastrointestinal fluid approaches that of the blood whether the original concentration of sodium sulfate is hypertonic or hypotonic. While our data include a few values of the total osmotic pressure which are close to those of the sera, most others are higher than the serum values. These apparent discrepancies from the literature could be due to differences in animal species and experimental conditions. Further inquiries in these areas are indicated.

Absence of evidence of irritation of the intestinal mucosa has been reported with sodium sulfate placed in the intestinal loops in the cat and dog (12) and also with cyclamates in the rat and dog after chronic administration (1, 2, 3). Similar observations with another saline cathartic, magnesium sulfate, were made in acute experiments in the cat (37) or after protracted use in the dog (38). No gross changes of the gastrointestinal mucosa were noted in the rat and mouse in the present experiments with the cyclamate and sodium sulfate.

The present experiments also show that the gastrointestinal tolerance of the cyclamate in the rat is not affected by the addition of saccharin (at 1/10 of the weight of the cyclamate; see TABLE XVI). This suggests that the possibility of reaching the limit of gastrointestinal tolerance to cyclamate is reduced when saccharin is added to double the sweetness ([1]).

([1]) Cyclamate in combination with saccharin at a ratio of 10 to 1 is used in most of the Sucaryl® Sweeteners of Abbott Laboratories.

Summary

The primary concern over the mechanism involved in the changes of the stool after large oral doses of the cyclamate prompted the present work in conjunction with parallel studies of a saline cathartic, sodium sulfate. The rat was chosen for most of the experiments; other species were also studied to supplement the evaluation. The rate of absorption of sodium sulfate, sodium cyclamate and calcium cyclamate as well as their apparent degree of ionization was determined. These values were used to determine the relative potency for the laxative response, the gastrointestinal motility and the osmotic retention of fluid in the alimentary tract of the rat. The effects of these salts were also studied on the isolated intestines of several species and the gastrointestinal motility and laxation in the mouse and dog. These studies led to the following conclusions: (*a*) Both the sulfate and cyclamate are partially absorbed after oral doses. About 60 % of the sulfate and 30 % of the cyclamate may be absorbed by the rat. (*b*) Both the sulfate and cyclamate have no significant systemic effect which contributes to the laxative action. (*c*) Both the sulfate and cyclamate increased gastrointestinal motility, liquidity of the stool and the bulk of fluid in the gastrointestinal lumen. All these are significantly correlated with the osmotic activity of the unabsorbed portion of the salt. There is no difference in the relative potency of these salts when considerations are given to the factor(s) of absorption and/or ionization. The cyclamate and sodium sulfate are therefore identical so far as their osmotic effect in the alimentary tract is concerned. Addition of saccharin to the cyclamate at a 1 to 10 ratio does not modify the laxative effect of large doses of the cyclamate in the rat.

Acknowledgments

I wish to express my deep gratitude to Dr. R. K. Richards for his continued interest and advice; to D. Asher for the radioactivity measurements and the initial absorption studies in the rat; to R. U. Robinson for the freezing point determinations; to F. Pohley for consultation on statistics; and to E. Godula, W. Schreiber and R. Wiseman for technical assistance.

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COMPARATIVE EFFECTS OF METHADONE, MEPERIDINE AND MORPHINE ON CONDITIONED SUPPRESSION

BY

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(Received for publication 27-10-1965).

The present report deals with comparative effects of potent analgesic and other centrally acting drugs on "conditioned suppression" which, presumptively, is one index of a conditioned emotional response (CER) in rats. The operations employed consisted essentially of producing suppression of a stable leverpressing response by repeatedly pairing an auditory stimulus with electric shock to the feet and testing for suppression under drug conditions. (As described in a previous paper (1), the present work is concerned only with performance of this CER and not with acquisition or extinction phases, nor with other forms of CERs based on aversive stimuli.) Although this technique has been given several labels other than "CER", it was initially developed to provide quantitative measures of anxiety (2, 3). Utilitarian and theoretical applications of this method have ranged from evaluation of electroconvulsive shock treatment in rats (4, 5), through evaluation of several drug actions (6–10), to its use as a tool in isolating some parameters which control emotional behavior (9, 11, 12).

A previous paper reported data on the effects of graded doses of morphine (1). It was found that at certain dose levels suppression of leverpressing was practically eliminated. Further work was then directed toward assessing the specificity of this effect by testing drugs with known morphine-like properties and other drugs which produce little or no clinical analgesia. The present study compares the actions of two other highly effective analgesic drugs (methadone and meperidine) with those of morphine. Results of studies on hypnotic, tranquilizing, analeptic and psychotomimetic drugs will be reported in a subsequent paper.

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with the control (grass pellets alone), the diet was offered *ad lib.* for 14 d to five sheep in a 5×5 Latin square design. DM intakes on the treatments, Na_2SO_4 in the diet, H_2SO_4 in the diet, Na_2SO_4 intraruminally and H_2SO_4 intraruminally, were 75%, 26%, 66% and 55% respectively of that on the control. Thus, H_2SO_4 decreased VFI more than Na_2SO_4 did, but the difference was much less when both were given intraruminally. Taste influenced intake of H_2SO_4, presumably because of the low pH of the treated pellets.

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Effects of dietary hydrochloric and sulphuric acids on voluntary feed intake and metabolism of sheep.

By J. L. L'Estrange and F. Murphy, *Department of Agricultural Chemistry, University College Dublin, Glasnevin, Dublin 9*

A. I. Virtanen in several publications, e.g. Virtanen (1933), claimed that silage preserved with H_2SO_4 was much less palatable than silage preserved with a mixture of HCl and H_2SO_4. This implies that excess dietary sulphate adversely affects the voluntary feed intake (VFI) of ruminants. Recent experiments on ammonium bisulphate and other sulphate salts with cattle (McCarrick, Gardiner, Poole, Spillane & Maguire, 1966) and sheep (L'Estrange, Clarke & McAleese, 1969) indicated that metabolic acidosis induced by the acid sulphate salts was more important in reducing VFI than sulphate *per se*.

The effects of dietary HCl and H_2SO_4 have now been compared direct in sheep. The acids, either singly or as a 50:50 mixture, were added to a pelleted grass diet to reduce the pH to 3·8 (80 ml 4 N-acid/kg grass pellets). Each diet, as well as the grass pellets alone, was offered *ad lib.* to eight sheep in a double 4×4 Latin square design for 20 d. The results are summarized in Table 1.

Table 1.

	DM-intake (kg/d) (day 0–20)	Wt gain (kg/d) (day 0–20)	Digestibility of DM (day 8–20) (%)	pH of rumen fluid*
Grass pellets only	1·46	0·168	54·5	6·51
Grass pellets + HCl	1·18	0·082	56·0	6·36
Grass pellets + HCl–H_2SO_4	1·18	0·052	56·0	6·44
Grass pellets + H_2SO_4	1·02	0·022	58·2	6·43
SE of treatment means	0·033	0·033	0·41	0·034

	Mean of samples taken from day 2–20				
	Blood pH	Plasma CO_2 (m-moles/l)	Plasma SO_4-S (mg/100 ml)	Plasma Cl (mg/100 ml)	Urine pH
Grass pellets only	7·46	26·4	6·32	372	8·07
Grass pellets + HCl	7·38	22·4	6·84	388	5·89
Grass pellets + HCl–H_2SO_4	7·40	23·0	7·58	394	5·78
Grass pellets + H_2SO_4	7·42	23·4	8·22	386	5·69
SE of treatment means	0·009	0·78	0·21	5·0	(–)

Values for DM-intake and wt gain are the means for eight sheep whereas the other values are the means for four sheep/treatment.
DM, dry matter.
*Mean value for samples taken four times daily on 2 d.

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Sulf

Dry-matter intake was reduced to 70% of that in the control treatment by H_2SO_4 and to 80% on both the HCl and the HCl–H_2SO_4 treatments. Live-weight gain of the sheep was correspondingly reduced. Rumen fluid pH was slightly lower but the digestibility of the diets slightly higher on each acid treatment. The acids caused a similar degree of metabolic acidosis but, whereas plasma sulphate was increased by dietary H_2SO_4, plasma Cl was not affected by dietary HCl.

The effect of HCl on VFI is ascribed to metabolic acidosis rather than to taste as intake decreased gradually up to day 4 on the treatment. The level of dietary sulphate was sufficiently high on the H_2SO_4 treatment but not on the HCl–H_2SO_4 treatment to reduce intake further.

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Transfer of metabolites between sheep by cross-circulation dialysis as a means of studying chemostatic control of voluntary food intake in ruminants. By J. M. FORBES, *Department of Agricultural Sciences, University of Leeds*

A survey of the literature suggests that 'chemostatic regulation of voluntary food intake is a distinct possibility' (Thye, Warner & Miller, 1970). Despite many attempts to elucidate such a control mechanism, both by infusion of substances intraruminally, intravenously or intracranially and by measurement of changes in rumen and plasma metabolites during a meal, chemostatic control has not yet been fully explained.

Parabiosis is a technique which has proved useful in the study of the regulation of food intake in rats (Fleming, 1969). It was not thought possible to join ruminants parabiotically and direct cross-circulation would involve the use either of identical twins, whose occurrence in sheep is not proved, or of immunosuppressive drugs which might affect energy metabolism.

These considerations have led to a development of a technique of cross-circulation dialysis (CCD) in which the blood of two animals is circulated independently through the two layers of a Kiil dialyser ('kidney machine'). Warm saline is recirculated over the cellophane membrane surfaces of the blood chambers and carries dialysed molecules along the concentration gradient between the blood-streams.

At first, sheep were equipped with semi-permanent carotid artery–jugular vein shunts but circulation through these could not be maintained for more than a few days, even when the animals were continuously infused with anticoagulants. Temporary polytetrafluoroethylene cannulas are now introduced into both jugular veins of each sheep and circulation is induced by peristaltic pumps. Heparin is used during CCD to prevent coagulation, and flow rates of 150 ml/min from each sheep are achieved. The duration of each CCD has so far been restricted to 4 h but runs of 24 h or more would appear to be quite feasible.

Ir. J. agric. Res. 8: 133-150, 1969

STUDIES ON HIGH INTAKES OF VARIOUS SULPHATE SALTS AND SULPHURIC ACID IN SHEEP

1. Effects on voluntary feed intake, digestibility and acid-base balance

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ABSTRACT

Five wethers in a Latin-square experiment were fed *ad lib* for 12 days and at a restricted intake for 11 days on pelletted grass meal alone or supplemented with either sodium sulphate, sodium bisulphate, ammonium bisulphate or ammonium sulphate, each in amounts equivalent to 1% sulphur in the dry matter. In a second experiment the five sheep were fed grass meal supplemented with sulphuric acid at the same level of sulphur for similar periods.

Sodium sulphate and sodium bisulphate decreased voluntary feed intake by 22%, and each of the other supplements decreased it by 44%. Reduction in intake was related to the degree of metabolic acidosis induced by the respective supplements, except for sodium sulphate. Feed intake returned to normal immediately after withdrawal of each supplement.

All supplements increased total sulphate concentration in blood serum, but did not affect dry matter digestibility or blood ammonia-nitrogen concentration. None of the treatments directly affected water intake, urinary volume or water content of faeces, except sulphuric acid which slightly decreased each. In the rumen the pH was reduced by sulphuric acid by 0.4 units, while ammonia-nitrogen concentration was increased by ammonium bisulphate and ammonium sulphate.

INTRODUCTION

Ammonium bisulphate has been found to be a good additive for ensilage of grass or grass-clover mixtures when added at a level of about 16 lb per ton of fresh herbage (1, 2). However, voluntary intake by cattle of ammonium bisulphate silage is much less than that of similar silage preserved with molasses (3, 4, 5), the reduction in intake being directly proportional to the amount of the salt added (6). Two suggestions have been put forward to explain the reduced voluntary intake. One is that ammonium bisulphate disturbs the acid-base equilibrium of the animals due to its acid bisulphate radical (7). McCarrick *et al* (7) observed that the salt induced metabolic acidosis in cattle as indicated by plasma CO_2 and urine pH values. They also showed (8) that sodium bicarbonate supplementation increased the voluntary intake of ammonium

bisulphate silage though never to the same level as that of corresponding molassed silage. Clifford (4) and Bryan (5) were of the opinion that reduced voluntary intake was caused by the high intake of sulphate *per se*. Their opinion would appear to be supported by the findings of other workers (9, 10). Virtanen (9) found that the consumption of silage preserved with sulphuric acid was very low compared with that of the same silage preserved with a mixture of hydrochloric and sulphuric acids; and Schmekel (10) showed recently that sodium bisulphate when used as a silage additive also decreased feed consumption of cattle which he ascribed to its high sulphur sulphate content.

The present series of experiments was undertaken to test the foregoing suggestions and also the third possibility that the ammonium ion in the salt may be a causative factor in reducing voluntary intake. In two experiments with sheep, the effects on voluntary intake and metabolism of sodium sulphate, sodium bisulphate, ammonium bisulphate and ammonium sulphate and of sulphuric acid, each added to provide 1% S to the feed dry matter, were compared.

EXPERIMENTAL

Animals

The sheep were 1- to 2-year-old Cheviot wethers, having a mean weight of about 40 kg. Throughout the experiments they were placed in metabolism cages (11).

Treatments

Experiment 1: Pelletted dried grass meal, similar in composition (Table 1) to that of good quality grass for ensilage, was used as the basal diet. Solutions (2 M) of each of

TABLE 1: Chemical composition of pelletted grass meal fed as basal diet

	% of dry matter
Crude protein	15.2
Ether extract	3.2
Crude fibre	29.2
Ash	9.5
Nitrogen-free extractives	42.9
K	2.90
Na	0.16
Ca	0.46
Mg	0.16
Cl	1.32
P	0.38
Total sulphur	0.41

the four sulphate salts — i) sodium sulphate, ii) sodium bisulphate, iii) ammonium bisulphate, iv) ammonium sulphate — were made up in distilled water. All remained in solution at room temperature, except sodium sulphate which had to be gently heated to dissolve it completely. Initially it was planned to feed each salt in two ways: a) by pouring the solution onto the basal diet daily before feeding; b) by drenching once daily a fixed amount of each salt. Drenching was discontinued on the first day after the sheep receiving ammonium sulphate (211 ml of a 2 M solution) died within 2 hours, apparently from ammonia toxicity. For the other technique batches of the experimental diets were made up as follows: 280 ml of each solution were poured slowly on to 2 kg of the grass meal pellets in a plastic container, which was shaken thoroughly to ensure an even mixture. As control, the grass meal pellets were diluted to the same extent with distilled water. Each batch of diets was fed within 3 days of being made up.

The five experimental diets were fed to five sheep in a 5 x 5 Latin-square experiment. The sheep were fed *ad lib* for 12 days, then on a restricted intake (equal intakes of each diet) for 11 days. This was followed immediately by a recovery period in which the sheep were fed the grass meal pellets alone *ad lib* for 6 days. Fresh distilled water was provided throughout and its intake recorded.

Experiment 2: In Experiment 2 all five sheep after completing Experiment 1 were fed the sulphuric acid treatment, *i.e.*, sulphuric acid added to the same basal diet as used in Experiment 1, also to supply 1% sulphur to the feed dry matter. This diet was fed *ad lib* for 12 days, then at the restricted intake for 11 days, followed by a recovery period of 1 day on the control diet *ad lib*.

Urine and faecal collections

Urine was collected in plastic buckets containing a small amount of toluene and petroleum ether (80° to 100°C), and its volume recorded daily. Two subsamples of each collection were taken for analysis; the one for bicarbonate analysis was stored under a layer of petroleum ether (80° to 100°C), and the one for the analysis of the other constituents was acidified with glacial acetic acid. Toluene was added to both subsamples. The faeces were collected throughout the experiment and samples were dried to constant weight at 80°C for subsequent analysis.

Blood and rumen sampling

All blood samples were taken from the jugular vein at 1- to 4-day intervals throughout the trial. Rumen samples were taken by the stomach pump technique, using a polythene tube connected to a motor vacuum pump and were preserved with mercuric chloride. For the control and sulphate salt treatments rumen samples were taken from one sheep only on each treatment. The samples were taken during the restricted intake period, which was extended for an extra 4 days to allow this sampling to be carried out

without interference with the balance trials also in progress. Samples were taken from all five sheep on the sulphuric acid treatment on the last day of the restricted intake period.

Analytical methods

All pH values were recorded with a Radiometer pH meter (type pH M26) using a glass electrode. For blood the pH and temperature were recorded simultaneously both immediately on sampling and after standing for about 30 minutes, on heparinised samples collected under petroleum ether. The pH values were corrected to 37°C (12). The pH of urine was measured daily on samples from the previous 24-hour collections. The pH of whole rumen contents was measured immediately after the sample was removed from the rumen.

The total CO_2 concentration in plasma and urine was determined by the method of Conway (13).

Ammonia-nitrogen in blood and urine was determined by the methods of Conway (13). For blood the sample was measured by syringe into the diffusion vessels immediately after sampling, and the corrections of Conway (13) were applied for the ammonia not released in the time allowed for diffusion (20 min). Ammonia-nitrogen in rumen fluid was measured by the method of Conway (13), using boric acid as the absorbing buffer and potassium metaborate in the outer chamber (14). Volatile fatty acids (VFA) in rumen fluid were determined by gas chromatography (15). Total sulphate sulphur was determined in plasma by the method of Dean (16), and total sulphur in feed samples by the method of Shaw (17).

RESULTS

The five sheep remained apparently healthy throughout the experiments and completed each period on the respective treatments. However, they all developed mild sores around the mouth while on the sulphuric acid treatment. Results of both experiments are presented and discussed together.

Dry matter intake

Dry matter intake was substantially reduced by all treatments relative to the control during the 12-day *ad lib* period. On both sodium sulphate and sodium bisulphate it was equal to about 78% of the control, while on ammonium bisulphate, ammonium sulphate and sulphuric acid treatments it was also equal, but only at about 56% of the control (Table 2). Despite reductions in intake there was no complete refusal of the diets by any sheep except for one animal which ate none of the sodium sulphate diet on the 1st day of treatment. Daily feed intake was almost constant on the control diet

TABLE 2: Effects of the sulphate salts and sulphuric acid on dry matter intake, dry matter digestibility and water metabolism of the sheep

	Experiment 1								Experiment 2	
	Treatment						se of treatment mean (mean of 5 sheep)	F-test[1]	Treatment	
	Control	Na_2SO_4	$NaHSO_4$	NH_4HSO_4	$(NH_4)_2SO_4$				H_2SO_4 (Mean ±se for 5 sheep)
Dry matter intake (g/day)									
a) 12 days *ad lib*	1,234a	958b	978b	729c	677c	±65.3	***	653±66
b) 11 days restricted intake period	591	602	596	579	576	± 7.1	NS	557± 9.7
c) 6 days recovery period on control diet *ad lib*	1,132	1,092	1,072	1,119	1,030	±43.6	NS	—
Dry matter digestibility (%)								
a) Last 6 days *ad lib* period	64.0a	67.9ab	68.1ab	74.1b	70.3ab	±1.96	*	69.3 ±0.80
b) Last 6 days restricted intake period	69.0	70.2	69.4	68.7	65.8	±1.29	NS	72.5 ±1.33
c) Whole trial including recovery period (29 days)	64.8	67.4	66.7	66.9	66.5	±0.60	NS	
Water intake (litres/day)								
a) 12 days *ad lib* period	2.95a	2.92a	2.76ab	2.17b	2.16b	±0.17	*	1.98±0.18
b) 11 days restricted period	2.30	2.46	2.29	2.06	2.35	±0.105	NS	2.18±0.14
Water excretion (ow for the whole 23 days)								
a) % H_2O in faeces	68.9	69.2	69.6	68.9	69.0	±1.69	NS	61.3 ±0.55
b) Vol urine (litres/day)	1.35	1.56	1.46	1.27	1.41	±0.089	NS	0.94±0.11

1 Where the F-test shows a significant treatment effect the means are significantly different from each other ($P<0.05$), if they do not have a letter in common

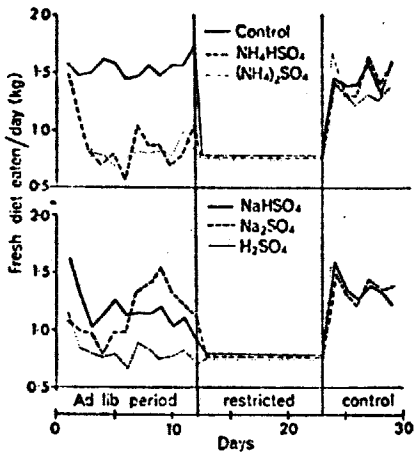

Fig. 1: Mean daily voluntary feed intake by the five sheep on each treatment. The dry matter content of each diet was about 78%. On the control diet during the recovery period it was 88%, but is corrected here to 78% for comparison. Intake is not expressed as weight of dry matter, because the moisture content of the residue was not analysed daily

during the *ad lib* period (Fig. 1). Intakes on sodium bisulphate, ammonium bisulphate and ammonium sulphate were about the same as on the control on the 1st day, but then gradually decreased until the 4th day after which they remained fairly constant on sodium bisulphate at about 76% of the control, and on ammonium bisulphate and ammonium sulphate at about 52% of the control. Daily intake on sulphuric acid was almost similar to that on ammonium bisulphate and ammonium sulphate except on the 1st day of treatment when it was considerably lower. The daily intake pattern was somewhat different on sodium sulphate. Intake was not as high on the 1st day as on the other treatments, partly due to complete refusal of feed by one animal. It further decreased daily until the 4th day, but from then on it increased again and over the last 6 days was about 87% of the control.

Dry matter intake during the restricted period was kept at the same level for all treatments, the level selected being a little less than that eaten on the treatment with the lowest intake during the *ad lib* period (Table 2). After withdrawal of the supplements there was no significant carry-over effect by any of the treatments on voluntary

feed intake. On the 1st day after each treatment intake of the grass meal pellets fed alone *ad lib* was approximately similar to that of the control diet during the 12-day *ad lib* period, and remained constant for the rest of the 6-day recovery period (Table 2 and Fig. 1).

Dry matter digestibility

Dry matter digestibility varied considerably for the last 6 days of both the *ad lib* and the restricted intake periods (Table 2). Digestibility measured over 29 days (*i.e.*, 23 days on experimental diets plus 6 days on the control diet) was much less variable and was slightly higher for the diets containing the sulphate salts (67%) than for the control (65%) (Table 2). Dry matter digestibility of the sulphuric acid diet measured over the whole feeding period plus the 1-day recovery period was 67.7%. The results show that none of the sulphate salts or sulphuric acid at the levels given reduced dry matter digestibility. The small increase which they caused may have been due to the higher digestibility of the salts added relative to the grass meal.

Water intake and excretion

During the 12-day *ad lib* period voluntary water consumption (Table 2) was lower on the ammonium bisulphate, ammonium sulphate and sulphuric acid treatments than on the control diet. During the restricted intake period water consumption on any of the sulphate treatments was not significantly different from that on the control diet, though it was highest on sodium sulphate and lowest on ammonium bisulphate. Intake of water, therefore, appears to have been more directly related to dry matter intake and only indirectly to any of the supplements added. Neither the water content of the faeces nor the volume of urine excreted was appreciably affected by any of the treatments, except sulphuric acid, which reduced both (Table 2).

pH of diets

The effect of the various supplements on the pH of the diets was measured using a mixture of 5 g of each diet and 50 ml distilled water. The results (Table 3) show that neither sodium sulphate nor ammonium sulphate affected the pH, whereas the sodium bisulphate and ammonium bisulphate supplements both decreased it from 5.4 to 4.0, and the sulphuric acid supplement further decreased it to 3.3.

Rumen pH, VFA and ammonia-nitrogen values

It should be noted that during the days of rumen sampling, the diet which was offered as a single feed at 10.00 a.m. was consumed within 2 hours on all treatments except on ammonium bisulphate where only half of it was eaten in that time. Because samples were taken from one sheep only per treatment in Experiment 1 it is not possible to separate statistically the treatment and animal differences.

TABLE 3: Means and standard errors of the pH of the diets and of the pH, ammonia-N concentration and total VFA in rumen contents

	Treatment					
	Control	Na_2SO_4	$NaHSO_4$	NH_4HSO_4	$(NH_4)_2SO_4$	H_2SO_4
pH of diets[1] (diluted 1 in 10 with distilled water)	5.40±0.037	5.48±0.046	4.02±0.094	4.01±0.077	5.41±0.040	3.34±0.044
pH of rumen contents[2]						
Before feeding	7.12±0.03	7.21±0.19	6.98±0.34	6.68±0.15	6.80±0.11	7.09±0.20
2 hr after feeding	5.93±0.13	6.09±0.18	5.97±0.29	6.39±0.30	6.22±0.16	5.57±0.24
4 hr after feeding	6.28±0.11	6.32±0.18	6.15±0.07	6.41±0.41	6.35±0.07	5.95±0.15
6 hr after feeding	6.30±0.18	6.23±0.44	6.05±0.12	6.10±0.14	6.04±0.01	6.12±0.20
Ammonia-N in rumen fluid[2] (*mg/100 ml*)						
Before feeding	22.5±3.0	16.3±2.6	17.3±1.3	19.6±3.6	18.7± 4.1	15.4±1.1
2 hr after feeding	23.1±3.4	20.7±3.4	23.0±1.6	36.2±4.2	24.6± 1.6	19.5±2.4
4 hr after feeding	23.8±4.2	23.2±1.6	23.4±3.8	26.1±1.5	50.7±11.3	22.5±4.0
6 hr after feeding	24.4±3.5	20.8±3.6	20.9±3.5	29.8±9.8	23.1± 3.5	21.0±3.8
Total VFA in rumen fluid[2] (*m moles/litre*)						
Before feeding	52± 6	61±3	64± 4	64±15	51±11	68±10
2 hr after feeding	94±14	97±6	83± 6	107± 1	96± 3	64± 6
4 hr after feeding	109±22	94±1	64±28	103± 5	108± 3	78± 5
6 hr after feeding	109± 6	83±1	93± 5	103± 2	89± 4	98± 4

1 Five samples per treatment
2 For all treatments except sulphuric acid the data on rumen fluid refer to samples taken from one sheep only per treatment, four samples before feeding, three at 2 and 6 hr after feeding and two at 4 hr after feeding; for the sulphuric acid treatment the data refer to one sample taken from each of the five sheep at each sampling time

TABLE 4: Means and standard errors of the molar percentage of the individual VFA in the rumen on each treatment[1]

		Treatment					
		Control	Na_2SO_4	$NaHSO_4$	NH_4HSO_4	$(NH_4)_2SO_4$	H_2SO_4
Acetic acid	Before feeding	62.0±1.8	70.8±0.9	68.2±3.3	68.8±4.0	65.5±2.6	65.1±3.3
	2 hr after feeding	53.0±1.1	64.7±2.4	59.3±2.4	65.7±2.7	62.0±0.6	61.0±2.1
	4 hr after feeding	55.0±2.0	64.5±2.4	67.5±4.4	67.0±3.9	62.5±0.5	61.2±1.0
	6 hr after feeding	62.5±5.4	66.0±1.0	64.0±1.0	60.7±3.8	62.3±3.8	63.5±1.7
Propionic acid	Before feeding	19.7±1.1	14.2±0.3	16.7±0.7	16.2±0.8	21.5±0.7	17.2±1.0
	2 hr after feeding	25.6±0.8	12.9±0.5	12.4±0.2	16.0±0.6	18.3±0.5	15.7±1.8
	4 hr after feeding	22.5±0.4	14.1±1.0	12.2±1.0	16.9±2.7	18.2±1.4	13.9±2.1
	6 hr after feeding	19.3±2.9	14.4±0.6	14.8±0.3	16.6±1.3	19.4±0.9	16.6±2.1
Isobutyric acid	Before feeding	4.5±0.4	2.4±0.3	5.3±0.6	6.4±1.0	2.3±0.6	4.5±0.8
	2 hr after feeding	6.1±1.5	7.8±1.1	7.3±1.1	4.5±0.9	4.7±1.0	8.7±1.6
	4 hr after feeding	5.0±0.3	4.3±0.1	5.5±1.4	3.8±1.4	3.6±1.3	6.7±2.4
	6 hr after feeding	4.4±1.1	4.2±0.5	4.6±0.8	5.2±0.4	4.3±0.8	4.4±0.7
Butyric acid	Before feeding	9.5±0.8	10.2±0.6	8.2±0.7	7.6±0.2	9.1±2.0	10.4±1.0
	2 hr after feeding	13.6±1.8	12.8±0.8	17.5±1.4	12.1±1.3	13.4±0.6	11.7±0.6
	4 hr after feeding	14.2±1.7	14.6±0.6	12.5±5.1	11.0±1.0	14.5±0.6	14.9±1.6
	6 hr after feeding	12.5±1.5	13.6±0.7	14.2±0.5	12.1±1.6	11.5±1.6	12.3±1.4
Isovaleric plus valeric	Before feeding	4.2±0.5	2.4±0.2	1.7±0.6	1.4±0.2	2.0±0.1	2.7±0.4
	2 hr after feeding	1.9±0.6	1.8±0.3	3.2±0.9	1.9±0.1	1.7±0.2	2.6±0.5
	4 hr after feeding	3.8±0.5	1.8±0.5	2.5±0.9	1.8±0.5	1.8±0.4	3.1±0.4
	6 hr after feeding	2.9±1.1	1.6±0.1	2.0±0.2	5.4±0.4	2.6±0.6	3.1±0.7

1 See footnote to Table 3 for details of samples

The effects of treatments on the pH of whole rumen contents (Table 3) would appear to have been small because, in the period 2 to 6 hours after feeding, only sulphuric acid produced values appreciably different from the control, *i.e.*, lower by about 0.4 units. The total VFA concentration (Table 3) was also lower on sulphuric acid than on the other treatments. The molar proportion of the individual VFA (Table 4) was almost similar on all treatments except that acetate was lower and propionate higher on the control than on the other treatments. This difference may have been an animal one, because when the five animals were sampled on the sulphuric acid treatment the sheep which had shown the high content of propionate on the control diet again had a similar higher content of propionate than the rest.

The observed values of ammonia-nitrogen concentration in rumen fluid were considerably higher on ammonium bisulphate at 2 hours after feeding and on ammonium sulphate at 4 hours after feeding than on the other treatments. Otherwise there were no marked treatment effects (Table 3).

Blood pH

The pH of whole blood (Fig. 2) remained constant on both the control and sodium sulphate treatments, average values being equal on both at 7.51. The values on sodium bisulphate treatment were slightly less throughout. On both ammonium bisulphate and ammonium sulphate the values decreased after the 1st day on each treatment to 7.40, then remained fairly constant at this low value throughout the experiment, but

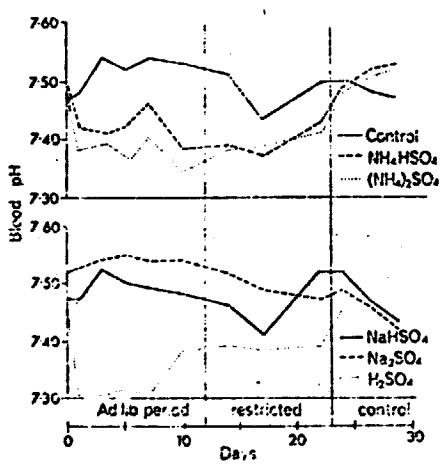

Fig. 2: Mean blood pH values of the five sheep on each treatment

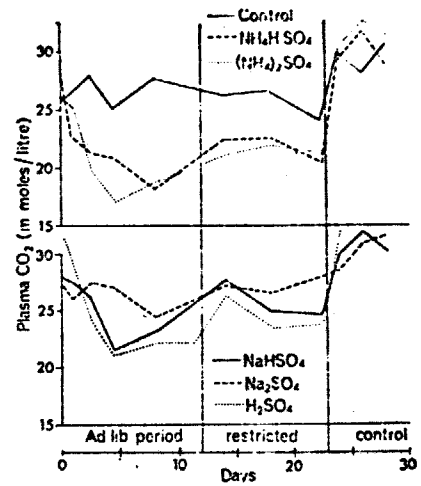

Fig. 3: Mean plasma CO_2 levels of the five sheep on each treatment

increased back to normal within 2 days of the change over to the control diet. The values on the sulphuric acid treatment changed in the same way as on the ammonium bisulphate and ammonium sulphate treatments, but were slightly lower throughout.

Plasma CO_2

The concentration of total CO_2 in blood plasma (Fig. 3) was constant on both the control and sodium sulphate treatments, mean values throughout the experiment being 26.9 and 26.7 m moles per litre respectively. The levels on the sodium bisulphate treatment were consistently lower, the mean value being 24.9, while on ammonium bisulphate and ammonium sulphate they were still lower, mean values being 21.2 and 20.6 m moles per litre respectively. On these three treatments the concentration decreased gradually until about the 4th day, then remained constant or increased slightly during the restricted intake period, and increased back to normal within 2 days of changing over to the control diet. The changes on the sulphuric acid treatment were almost similar to those on ammonium bisulphate and ammonium sulphate, though the concentrations were not quite so low, mean value being 23.4 m moles per litre.

Results of blood pH and plasma CO_2 measurements indicate that there was a mild degree of metabolic acidosis caused by the sodium bisulphate treatment, and a more severe form, but similar for each, caused by the ammonium bisulphate, ammonium sulphate and sulphuric acid treatments.

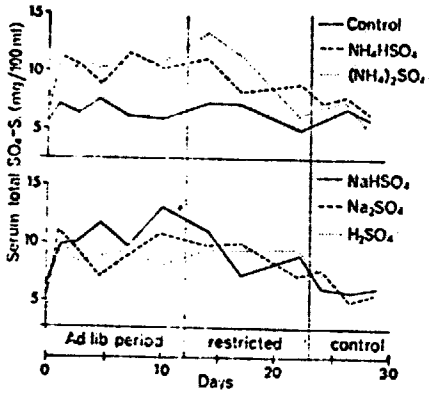

Fig. 4: Mean levels of total sulphate sulphur in serum of the five sheep on each treatment

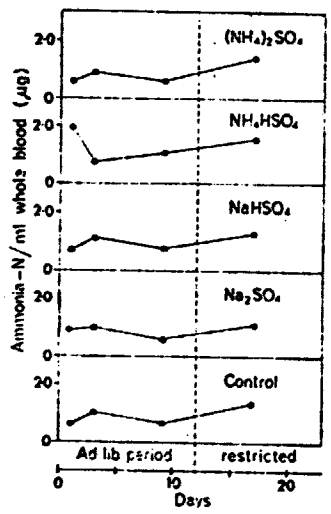

Fig. 5: Individual values for whole blood ammonia-nitrogen obtained from one sheep per treatment

Total sulphate sulphur in serum

The concentration of total sulphate sulphur in serum was almost similar on the four sulphate salt treatments and on the sulphuric acid treatment, at about 10 mg per 100 ml compared with 6.5 mg per 100 ml on the control diet. The high values were reached within 2 days on each treatment; the concentrations then remained fairly constant, but decreased to the original levels within 2 days of changing over to the control diet (Fig. 4).

Blood ammonia-nitrogen

Data obtained from the analysis of a small number of samples showed no apparent differences in the concentrations of blood ammonia-nitrogen between treatments (Fig. 5). None of the values was high, the average being about 0.9 µg per ml.

Urine pH

The pH of urine (Fig. 6) was constant throughout at about 8.1 on both the control and sodium sulphate treatments. On each of the other four treatments it decreased to about 6.0 within 4 days, and remained at this value until the supplement was withdrawn. It then increased to 8.0 within 3 days.

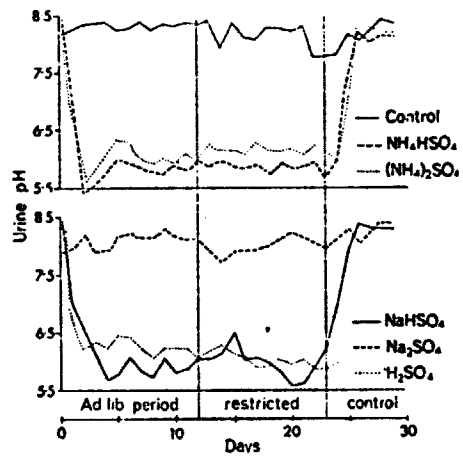

Fig. 6: Mean daily pH values in urine of the five sheep on each treatment

Urinary bicarbonate and ammonia-nitrogen excretions

Bicarbonate excretion in urine (Fig. 7) was similar on the control and sodium sulphate treatment at about 270 m moles per day throughout the experiment, with some

reduction during the restricted intake period. On each of the other treatments bicarbonate excretion decreased gradually to reach a constant level of about 40 m moles per day within 6 days. Excretion increased again to the levels on the control diet within 4 days of withdrawal of the supplements. Excretion of ammonia-nitrogen in urine was constant at about 17 m moles per day on both the control and sodium sulphate treatment, but was about twice that level on sodium bisulphate (Fig. 8). On each of the other treatments ammonia-nitrogen excretion increased to about 150 m moles per day within 4 days. It decreased again to the level on the control diet within 4 days of withdrawal of the supplements. These data along with those on blood pH and CO_2 show that the grass meal alone was alkaline to the animals. The sodium bisulphate supplement was sufficient to make the diet slightly acidic. The ammonium bisulphate, ammonium sulphate and sulphuric acid supplements made the diet more acidic to the animals, the degree of acidity being equal for all three, showing that the ammonium ions of ammonium bisulphate and ammonium sulphate were equally as acidic to the sheep as the hydrogen ions of ammonium bisulphate and sulphuric acid.

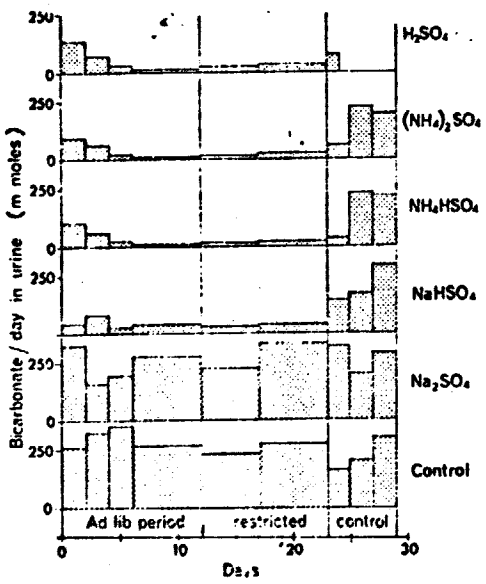

Fig. 7: Mean output of bicarbonate in urine of the five sheep on each treatment

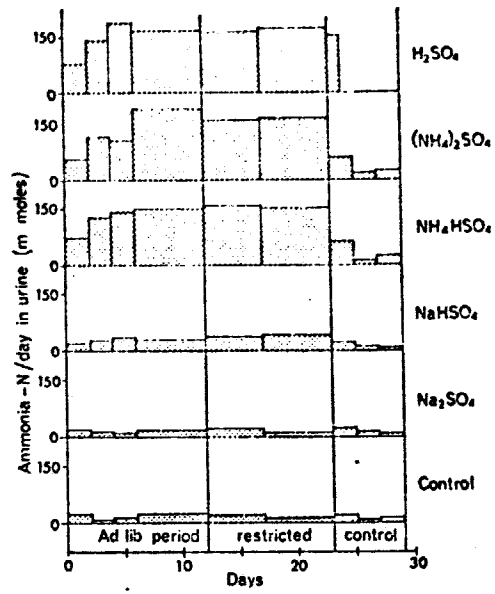

Fig. 8: Mean output of ammonia-nitrogen in urine of the five sheep on each treatment

DISCUSSION

In these experiments, with sheep, ammonium bisulphate added to a pelletted grass meal ration at a level supplying 1% sulphur to the feed dry matter reduced voluntary intake of the grass meal by about 44%. This reduction is similar to that caused in cattle by an equivalent level of ammonium bisulphate added to herbage for ensilage (4, 6). Two conclusions can be tentatively drawn from this result:

a) that sheep are affected in the same way as cattle by ammonium bisulphate in their diet; and

b) that the effect of ammonium bisulphate on feed intake, where it is used as a silage additive, is a direct effect of the salt on the animal and not an indirect one associated with the type of preservation produced in the silage by the salt.

Ammonium sulphate, sulphuric acid and ammonium bisulphate, when added to the grass meal ration to supply the same level of sulphur, each caused a similar reduction in feed intake, whereas sodium bisulphate caused about half that reduction. On

all four treatments the voluntary feed intake was not affected on the 1st day after introduction of the supplement. It then decreased gradually until the 4th day after which it remained constant. This indicates that the effect of these compounds on intake was associated not with their palatability but rather with a metabolic disturbance of the animals. Sodium sulphate also decreased the voluntary intake of the grass meal to the same extent as did sodium bisulphate over the 12-day feeding period, but its effect was more pronounced in the first few days of feeding and less after that, indicating some differences in its mode of action.

The effects of the four sulphate salts and of sulphuric acid on the concentration in serum of total sulphate sulphur were almost similar, *i.e.*, a sharp increase within 2 days to a constant level and then a sharp decrease again when each compound was withdrawn. Serum inorganic sulphate sulphur concentration has previously been shown to vary in a similar way with the sulphate intake of sheep (18). There was no major treatment effect on water intake, urine volume or water content of faeces except that all were slightly lower on sulphuric acid. The effects of treatments on the pH of rumen contents appeared to be small, the most noticeable being a reduction of 0.4 units caused by sulphuric acid. None of the treatments reduced feed dry matter digestibility or greatly altered rumen VFA production. It can be concluded, therefore, that none of the foregoing factors was the main cause of the reduction in feed intake.

The effect of sodium sulphate on feed intake was probably caused by the high intake of sulphate, as it has previously been shown (19) that much higher levels of sodium fed as sodium chloride did not reduce the feed intake of young lambs. The greater reduction in intake caused by ammonium bisulphate, ammonium sulphate and sulphuric acid was clearly due not only to the sulphate ion but also to the ammonium and the hydrogen ions. Since the reduction in intake was similar for all three compounds the hydrogen and ammonium ions had an equal effect on intake. Following the introduction of these three compounds the reductions in intake were clearly related to the development of metabolic acidosis as indicated by blood pH and CO_2 values and by urinary pH and ammonia-nitrogen excretion. It is concluded that this metabolic acidosis was the main cause of the reduced feed intake. The reduction in intake caused by sodium bisulphate could be ascribed to its sulphate ion, though its hydrogen ion appeared to accentuate the sulphate effect especially after the first 6 days.

Because the degree of metabolic acidosis was similar for ammonium bisulphate, ammonium sulphate and sulphuric acid, it is apparent that the ammonium ions in both ammonium bisulphate and ammonium sulphate were acidic to the animals when absorbed, being metabolised to free ammonia and, thereby, releasing hydrogen ions. In the same way ammonium chloride is well known to be acidic to non-ruminants (20) and ruminants (21). Consequently, metabolic acidosis in cattle induced by the feeding of ammonium bisulphate silage is caused not only by the bisulphate radical as suggested by McCarrick *et al* (6), but also by the ammonium ion. Although ammonium bisulphate and ammonium sulphate increased the concentration of rumen ammonia-

nitrogen they did not increase the blood ammonia-nitrogen concentration above normal values reported by Lewis (21). This is further evidence that their effect on feed intake was caused by way of metabolic acidosis rather than by direct ammonia toxicity, such as has been reported in sheep drenched with large amounts of either ammonium chloride, ammonium acetate or urea (21). However, in a preliminary trial, a sheep given by drench an amount of ammonium sulphate equal to that supplied by 750 g of the supplemented diet developed symptoms of ammonia toxicity and died within 2 hours. A second sheep drenched with an equivalent amount of ammonium bisulphate also developed symptoms of ammonia toxicity, but recovered. It is very likely that ammonia toxicity would be more easily induced by drenching these salts than by feeding them incorporated in the diet, in the same way as urea given by drench is much more toxic than when given with the whole diet (14).

As far as we are aware, the effects of ammonium sulphate on metabolic acidosis and reduction of feed intake in sheep shown here have not previously been reported. This compound has been observed to be a good non-protein nitrogen source for protein synthesis by rumen micro-organisms in *in vitro* studies (22), but unless it can be fed with appropriate alkaline salts it is unlikely to be a useful non-protein nitrogen source for ruminants.

Finally it is concluded from this experiment that sodium bisulphate should be a more useful silage additive than ammonium bisulphate, since both had an equal effect on the lowering of the pH of grass meal, but ammonium bisulphate had a more adverse effect on metabolic acidosis and reduction of feed intake. Also, sulphuric acid should be more useful than ammonium bisulphate since it was more effective in lowering the pH of the grass meal, and had an equal effect on metabolic acidosis and reduction of feed intake.

ACKNOWLEDGMENTS

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Alimentary, blood and other changes due to feeding $MnSO_4$, $MgSO_4$ and Na_2SO_4[1]

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MOINUDDIN, JESSIE FISCHER AND HELEN WING-TSIT LEE. *Alimentary, blood and other changes due to feeding $MnSO_4$, $MgSO_4$ and Na_2SO_4.* Am. J. Physiol. 199(1): 77-83. 1960.—Rats were fed a basal diet or diets containing extra $MnSO_4$, $MgSO_4$ or Na_2SO_4 in three experiments. The major effects occurred in the experiment with the highest level of extra sulfates (138 mmoles/kg of feed) as follows. The $MgSO_4$-fed rats ate less feed than the Na_2SO_4-fed rats. Also, in the $MgSO_4$ group the activity of serum alkaline phosphatase was greater and the cleaned, dried cecum was considerably heavier than in the other groups, and the colon plus rectum was slightly longer than in the basal and $MnSO_4$ groups. The $MnSO_4$-fed rats, compared to rats of the other groups in this experiment, ate less feed, gained less body weight, required more feed per gram of gain, drank more water, voided more urine, showed depigmentation of the labial surfaces of the incisor teeth, had less hemoglobin and serum inorganic phosphorus, and had cleaned, dried stomachs of lighter absolute weight. The erythrocyte count was higher in the $MnSO_4$ group than in the basal or Na_2SO_4 groups.

CERTAIN SUGARS IN THE DIET stimulate the growth of various regions of the gastrointestinal tract in rats. For example, diets containing 67% glucose or 67% sucrose, as compared to diets containing 67% cornstarch, increase the dry weight of the cleaned stomach (1). Diets containing 25% lactose (2) or 67% sucrose (1) increase the dry weight of the small intestine, and diets containing 15% cellobiose (1) or 10% arabinose (2) show some evidence of doing the same. Also, the 25% lactose (2), 10% arabinose (2) and 15% cellobiose (1) diets increase the dry weight of the cecum and of the colon plus rectum. These results, considered along with the number of sugar particles per unit weight of feed in each diet and with the digestibility or absorbability of each sugar, led to the idea that the increases produced in the growth of each organ by the designated sugars might be related to the osmotic effect of sugar particles in the lumen. However, results of feeding antibiotics indicated that microorganisms in the gut might play at least some part in some of the changes in gut weights. Thus, conceivably the sugars might be stimulating gastrointestinal growth in some way by serving as ready substrates for fermentation by the microflora of the lumen. Although we realized that there could be still other explanations of the stimulatory effects of dietary sugars, yet it seemed worthwhile to try to distinguish between osmotic effects of sugars and effects on microflora of the tract as possible causes of the stimulatory actions.

An indirect approach used in the present work was to see if gastrointestinal growth in the rat was increased by feeding substances (other than sugars) which have considerable osmotic effects in the lumen. For this purpose, we chose the hydragogue cathartics, magnesium sulfate and sodium sulfate. Manganese sulfate was tested also, since manganese has been found to stimulate the growth of the small intestine and of the ceca in chicks (3). It seemed unlikely that any of these three sulfates would affect the microflora of the lumen in the same way that the sugars might. However, some other effect of one or more of these salts on the microflora could not be ruled out. Also, there would be other uncertainties in the interpretation of any stimulation of growth by hydragogue cathartics. Thus, the present experiment was exploratory rather than a clear-cut test of alternatives. Other approaches to the problem are needed also.

METHODS

In three separate experiments, weanling male Sprague-Dawley rats were housed in individual cages and given tap water and their respective diets ad libitum for 4 weeks. In each experiment, there were six rats in each of the four dietary groups. In *experiments a* and *c*, feed intakes and feed:gain ratios were obtained for each week and for the total 4-week period. In all three experiments, records were kept of any diarrhea which occurred. The rats were weighed at the beginning of the 4-week period, at the end of each week, and at the end just before killing them. The rats were killed as described previously (2)

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over a 3-day period. The gastrointestinal organs were freed from mesentery and excised. The small intestine and the colon plus rectum were hung full length, each with a 5-gram weight attached to the lower end. When these organs had straightened out completely, the lengths were measured. The organs were cleaned, examined, dried and weighed (2). In *experiment c*, a small snip of stomach was removed (for histological examination) before drying the organ, so that the dry weights of the stomach do not reflect the true weights as accurately as in *experiments a* and *b*.

In *experiment c*, additional observations were made as suggested by certain developments during the feeding period. These included visual observation of the teeth and of the volume of urine. Also, the amount of water drunk during the first 48 hours of the 3rd week was recorded. After 3½ weeks of the feeding period, blood was obtained from the tail for red and white blood cell counts and for determination of hemoglobin by the oxyhemoglobin method. At the time of killing the rats, blood was collected from the severed great vessels in the neck, and the serum from this blood was analyzed for alkaline phosphatase (4), inorganic phosphate (5), and protein (6). Many tissues were preserved for later analysis and examination, but it has not yet been possible to arrange for completion of this part of the study.

The basal diet, designated as the cornstarch diet, in all three experiments contained 67% cornstarch (Buffalo brand no. 3401, generously donated by the Corn Products Sales Co., New York City), 24% 'vitamin-free' casein, 5% Crisco, 4% salt mixture (U.S.P. XIV), and vitamins as given in detail elsewhere (7). The salt mixture was purchased from the Nutritional Biochemicals Corp., and it contained 3.8% magnesium sulfate (anhydrous) and 0.02% manganous sulfate monohydrate, but no sodium sulfate. For the diets high in sulfates of manganese, magnesium and sodium, part of the cornstarch of the basal diet was replaced, respectively, by extra manganous sulfate (monohydrate, Mallinckrodt, analytical reagent, A.C.S.), extra magnesium sulfate (anhydrous powder, Mallinckrodt, analytical reagent), or sodium sulfate (anhydrous powder, Merck, A.C.S.). In *experiment a*, the amount of the respective sulfate salt added, over and above that in the salt mixture, for the $MnSO_4$, $MgSO_4$ and Na_2SO_4 groups was 0.88 mmole/kg. This level was fed throughout the experiment. In *experiment b*, the respective sulfates were added initially at a level (again, over and above that in the basal diet salt mixture) of 8.64 mmole/kg of feed. This level was progressively increased at the same times for the $MnSO_4$, $MgSO_4$ and Na_2SO_4 groups in *experiment b* by doubling it on the 9th day of the feeding period to 17.28 mmole/kg, doubling this on the 17th day to 34.56, doubling the latter on the 25th day to 69.12 and there maintaining the level for the remaining 4–6 days. The final percentage by weight in the diet of the major sulfate constituent (now including the contributions of the basal salt mixture) was 1.2% $MnSO_4 \cdot H_2O$, 1.0% $MgSO_4$ and 1.0% Na_2SO_4 for the respective high sulfate diets in *experiment b*. In *experiment c*, the extra sulfates were added at the level of 138 mmole/kg of feed throughout the experiment.

In *experiments b* and *c*, the numerical results were analyzed statistically by analysis of variance, the hypothesis in every case being that the groups were equal and the alternative that they were not equal. In those cases where the F value indicated that there was a difference among the groups at the 5% level or less, a multiple range test (8) was applied to determine which among the groups differed from any other. The results of the latter test are indicated in the tables for the 5% and 1% levels of probability, and are referred to as significant in text statements if $P < 0.05$.

RESULTS

No data are presented for *experiment a*, since, at the low levels of sulfate salt additions in this experiment, all quantities examined were similar in all groups. Also, there were no significant differences among the groups in body weight gains within *experiment b*, so these gain data also are omitted.

Gains in body weight, intakes of feed and water, and feed : gain ratios (table 1). In *experiment c*, the rats in the $MnSO_4$ group, as compared to the rats in the other groups, gained less weight, ate less feed, required more feed per gram of gain and drank much more water. Although the $MgSO_4$-fed rats suffered chronic diarrhea (see later), they did not drink more water than the rats fed the cornstarch or Na_2SO_4 diets. The $MgSO_4$-fed rats ate significantly less feed than the Na_2SO_4-fed rats; they tended to eat less feed than the cornstarch diet rats also, but this latter tendency was significant only in the 1st week of the experiment. Thus $MgSO_4$ resembles another magnesium salt, $MgCl_2$, which also depresses the intake of feed when fed at excessive levels (9).

Volume of urine. In *experiment c*, rats in the $MnSO_4$ group voided more urine than rats in the other groups, as shown by much larger pools of urine on the waxed papers placed beneath the rats. The increase in volume

TABLE 1. *Gains in Body Weight, Intakes of Feed and Water, and Feed: Gain Ratios in Experiment c*

Dietary Group	Gain in 4 Wk.	Feed Intake in 4 Wk.	Over-All Feed: Gain Ratio for 4 Wk.	Water Intake, 1st 48 Hr of 3rd Wk.
	gm	*gm*	*gm/gm*	*ml*
Cornstarch	186 (178–199)	386 (375–396)	2.08 (1.97–2.16)	48 (36–58)
$MnSO_4$	109* (101–124)	301* (280–319)	2.76* (2.52–2.89)	83* (53–99)
$MgSO_4$	180 (162–195)	356† (324–392)	1.98 (1.84–2.12)	46 (37–55)
Na_2SO_4	192 (180–209)	408 (371–453)	2.13 (2.03–2.19)	51 (40–60)

Figures given are averages; ranges are in parentheses. Average b. wt. at start was 55 gm in each of the groups. * Significantly different from the other 3 groups ($P < 0.01$). † Significantly different from the Na_2SO_4 group ($0.01 < P < 0.05$).

was estimated as at least twofold, but exact measurements were not made.

Teeth. During the 3rd week of *experiment c*, we noticed that the front surfaces of the incisor teeth of five out of six rats in the $MnSO_4$ group were white or very pale yellow in color. Only one rat in the $MnSO_4$ group had incisors of the normal orange-yellow color, whereas every rat in each of the other groups had normally colored incisors. These differences persisted until the end of the experiment.

Blood findings in experiment c (table 2). Hemoglobin in the $MnSO_4$ group was significantly lower than in the other three groups. Only one rat in the $MnSO_4$ group had a hemoglobin content falling within the range of the other three groups; this was the same rat which had normally colored incisor teeth. The numbers of red blood cells were significantly greater in the $MnSO_4$ group than in the cornstarch or Na_2SO_4 groups. The differences among the groups in numbers of white blood cells were not significant statistically. The differences in serum protein were not significant; however, three values in the $MnSO_4$ group were slightly below the normal range of 6.2–7.1 gm/100 ml (10). The $MgSO_4$ group exceeded all other groups in serum alkaline phosphatase activity. Serum inorganic phosphate was lower in the $MnSO_4$ group than in the other groups, in agreement with some other work in which fairly high levels of manganese were fed to growing rats (11, 12).

Diarrhea. In *experiment b*, there were no cases of diarrhea in the cornstarch group, one slight case in the $MnSO_4$ group and two slight cases in the Na_2SO_4 group lasting only 1 day each. In the $MgSO_4$ group, two of the six rats had no diarrhea, three had only slight diarrhea (one for 1 day, another on 2 nonsuccessive days, and the 3rd on 3 nonsuccessive days), while the remaining rat had slight diarrhea on 6 different days (including 4 in succession) during the middle periods of the experiment and moderate diarrhea for the 3 successive days following the last increase in $MgSO_4$ level. Also, on certain other days when diarrhea did not occur, the feces of this rat and of one other rat in the $MgSO_4$ group were soft and had a variety of unusual shapes: some pellets were extra long but of normal thickness, some were very thin but of normal length, and some showed the lines of 'working over' plainly. Also, several of the pellets had several small sideways projections at one end and thus resembled cloves. The pellets were black in all groups.

In *experiment c*, there was no diarrhea in the $MnSO_4$ group and only one instance of slight diarrhea lasting 1 day in the cornstarch group. Only one rat in the Na_2SO_4 group had any diarrhea at all. This occurred on 4 different days (including 3 in succession) in about the middle of the feeding period, and was slight in each instance. Diarrhea began in all the rats of the $MgSO_4$ group after 1–3 days of commencing the feeding, and continued until the end of the experiment. Once established, diarrhea in this group was severe on most days during the first 3 weeks, and slackened in severity slightly in some of the rats during the 4th week. However, there was no clear-cut indication of remission of the $MgSO_4$-produced diarrhea, such as occurs with the diarrhea produced by various sugar-containing diets (1, 2, 7). Haag and Palmer (13) also found that marked catharsis continued throughout many weeks of an experiment when rats were fed diets containing 0.8–0.9% magnesium as either the sulfate or the carbonate and amounts of calcium and phosphorus similar to those in the diets herein.

Appearance of organs. In *experiment b*, the cecal contents in the $MgSO_4$ group were quite runny (although the liquid contained some solid material) as compared to the more pasty or waxy cecal contents in the other groups. In the $MgSO_4$ group of *experiment c*, the contents of the cecum and colon were runny, and the cecum was noticeably enlarged compared to the cecum in the other groups. The tissues around the rectum in the $MgSO_4$ group seemed to be tougher and more difficult to separate from the rectum than is usual, perhaps indicating that some adaptation occurred external to the organ itself.

Weights and lengths of organs (table 3). In *experiment b*,

TABLE 2. *Blood and Serum in Experiment c*

Dietary Group	Tail Blood After 3½ Weeks of Feeding the Diets			Serum From Blood From Vessels in Neck, Obtained at Killing		
	Hemoglobin	Red blood cells	White blood cells	Protein	Alkaline phosphatase	Inorganic phosphate
	gm/100 ml	*millions/cu mm*	*thousands/cu mm*	*gm/100 ml*	*Bodansky U/100 ml*	*mg P/100 ml*
Cornstarch	14.4 (13.2–16.3)	6.91 (6.00–7.60)	9.19 (1.75–15.45)	6.7 (6.4–6.9)	84 (73–91)	5.3 (4.4–6.2)
$MnSO_4$	11.6* (10.0–14.0)	8.80† (7.40–11.00)	16.21 (11.95–21.20)	6.3 (5.7–7.0)	85 (81–89)	3.8§ (3.6–3.8)
$MgSO_4$	14.6 (14.1–15.4)	7.74 (6.90–8.75)	12.23 (6.70–16.60)	6.8 (6.6–7.0)	99‡ (90–106)	6.0 (4.8–7.0)
Na_2SO_4	14.6 (14.0–15.2)	6.94 (5.85–8.05)	11.72 (2.20–23.40)	6.5 (6.3–6.8)	78 (66–83)	5.3 (4.8–5.8)

Values given are averages; ranges are in parentheses. Six rats per group, except that the values and statistical analyses for phosphatase and phosphate are for 4 of the 6 rats in each group. The determinations of phosphatase in the sera of the other 2 rats of each group were ruined. The pooled sera of these remaining 2 rats of each group yielded values of 6.1, 3.7, 6.3 and 5.8 mg P/100 ml for the cornstarch, $MnSO_4$, $MgSO_4$, and Na_2SO_4 groups, respectively. * Significantly different from the other 3 groups ($P < 0.01$). † Significantly different from cornstarch and Na_2SO_4 ($0.01 < P < 0.05$). ‡ Significantly different from Na_2SO_4 ($P < 0.01$) and from cornstarch and $MnSO_4$ ($0.01 < P < 0.05$). § Significantly different from $MgSO_4$ ($P < 0.01$) and from cornstarch and Na_2SO_4 ($0.01 < P < 0.05$).

TABLE 3. *Body Weight, Weights of Cleaned, Dried Organs and Length of Small Intestine and Colon Plus Rectum in Experiments b and c*

Dietary Group	B. Wt.	Weights of Cleaned, Dried Organs				Lengths of Organs	
		Stomach	Small int.	Cecum	Colon+rectum	Small int.	Colon+rectum
	gm	*mg*	*mg*	*mg*	*mg*	*cm*	*cm*
			Experiment b				
Cornstarch	211 (193–235)	214 (196–235)	945 (877–996)	65 (52–74)	144 (130–164)	99 (96–105)	17.2 (16.5–18.5)
$MnSO_4$	210 (187–234)	231 (211–243)	904 (760–993)	73 (64–84)	150 (133–163)	100 (95–112)	17.6 (16.5–19.5)
$MgSO_4$	216 (171–237)	222 (184–244)	922 (844–1007)	80* (63–90)	145 (124–166)	98 (91–103)	17.8 (17.0–18.5)
Na_2SO_4	211 (172–246)	218 (189–247)	875 (798–1029)	63 (51–75)	142 (125–162)	96 (87–101)	17.1 (15.5–18.5)
			Experiment c				
Cornstarch	248 (232–260)	222 (202–238)	1011 (881–1174)	82 (70–97)	162 (145–184)	104 (99–107)	17.4 (16.5–19.0)
$MnSO_4$	171† (156–190)	179‡ (139–210)	915 (828–1025)	68 (60–75)	157 (141–180)	101 (94–105)	17.4 (15.5–19.0)
$MgSO_4$	243 (228–260)	217 (198–236)	1029 (961–1116)	161† (140–183)	165 (147–201)	105 (98–111)	19.7§ (18.5–21.0)
Na_2SO_4	256 (236–281)	229 (213–244)	1047 (1000–1134)	80 (71–90)	156 (144–167)	107 (104–112)	18.0 (16.0–19.5)

Values given are averages; ranges are in parentheses. Various groups are compared only within each experiment. In *experiment c*, weight of stomach is low in all groups due to removal of a small sample for histological examination. In *experiment c*, weights of small intestine were found to be different by analysis of variance ($0.01 < P < 0.05$), but no differences between single means were found using the multiple range test. * Significantly different from Na_2SO_4 and cornstarch ($0.01 < P < 0.05$). † Significantly different from the other 3 groups ($P < 0.01$). ‡ Significantly different from cornstarch and Na_2SO_4 ($P < 0.01$), and from $MgSO_4$ ($0.01 < P < 0.05$). § Significantly different from cornstarch and $MnSO_4$ ($0.01 < P < 0.05$).

the cecum was significantly heavier in the $MgSO_4$ group than in the cornstarch and Na_2SO_4 groups, but the differences were not very large.

In *experiment c*, the $MnSO_4$ group was exceeded by the other three groups in final body weight and in the absolute weight of the stomach. The absolute weight of the small intestine also tended to be least in the $MnSO_4$ group. However, the $MnSO_4$ group significantly exceeded the other three groups in the weight of the stomach and small intestine in relation to body weight (not given in table 3). The $MgSO_4$ group exceeded the other groups in the absolute and relative weights of the cecum. $MnSO_4$ exceeded Na_2SO_4 in relative cecal weight (not given in table 3). The absolute weights of the colon plus rectum did not differ significantly among the groups, but the relative weights of this organ (not given) were significantly greater in the $MnSO_4$ group than in the other groups. Most of the differences in weights of organs in *experiment c* appear to be related to the smaller size and body weight attained by the rats in the $MnSO_4$ group; there are difficulties in interpreting differences in weights of organs in such a situation. Thus, while the absolute weights of the small intestine, the cecum and the colon plus rectum in the $MnSO_4$ group were lowered less drastically than was the body weight, we don't know if the growth of these intestinal organs is stimulated by $MnSO_4$ or if it simply is not poisoned as greatly by $MnSO_4$ as is the growth of the body in general. The only clear-cut, interpretable effect of diet upon weights of organs in *experiment c* is the large increase in the weight of the cecum in the $MgSO_4$ group.

In *experiment c*, the colon plus rectum was longer in the $MgSO_4$ group than in the cornstarch or $MnSO_4$ groups. The ratio of weight to length of this organ (not given in table 3), like the absolute weight, did not differ significantly among the groups. Many other dietary substances (agar, for example) increase the colonic weight and weight:length ratio, as well as the length, in relation to the cornstarch group (14). Magnesium sulfate is the first substance we have found, among those which have any effect at all on the colon plus rectum, which appears to increase only the length.

DISCUSSION

Effects of $MnSO_4$. Most of the significant differences among the groups at the highest level of sulfates fed (that is, in *experiment c*) were due to the $MnSO_4$ group differing from the other three groups. These differences probably were due to manganese rather than to sulfate, since the $MgSO_4$ and Na_2SO_4 diets contained the same amounts of sulfate as did the $MnSO_4$ diet, and since more of the $MgSO_4$ and Na_2SO_4 diets were eaten. However, it remains possible that sulfate may affect the interaction of manganese and other elements.

We found that manganese, at the highest level we fed, caused decreases in growth, in intake of feed and in efficiency of conversion of feed to gain in body weight. Others have also reported that manganese in excess depresses the growth of young rats (11, 12, 15–17). Several changes possibly may be involved separately in causing this slow growth. First, an increased loss in the feces of phosphorus resulted from feeding excessive amounts of manganese (11), and low levels of inorganic phosphorus were found in the serum of manganese-fed young rats when the diets contained about 0.25% (12), 0.36% (11) or 0.47% (present paper) of phosphorus. Raising the dietary level of phosphorus to 0.80% raised the level of serum inorganic phosphorus and markedly improved growth (11). Second, the slowness of growth may have been related, in part, to the anemia caused by excessive manganese (17, present paper). Third, depletion of the rat's bodily thiamine by manganese and fourth, some alteration of energy metabolism mediated through the

thyroid gland are possible factors suggested by the work of others but regarding which we have no information from the present experiments.

Increases in the intake of water and in the volume of urine appear not to have been reported previously as symptoms of toxicity of manganese. In regard to possible causes of these increases, it may be pertinent that a somewhat prolonged decrease in the level of serum magnesium occurred in dairy calves fed supplemental manganese in experimental diets (18), and that a temporary decrease in serum magnesium occurred in cows grazing pastures of high manganese content and in rabbits, sheep or cows given manganese salts (19). Also, the volume of urine increased during magnesium deficiency in the rat (10). So it seems possible that excessive manganese increased the volume of urine by interfering with the absorption or metabolism of magnesium, thus inducing a deficiency of magnesium. Or possibly, since excessive manganese disturbs the iron picture (see later), the diuretic effect of $MnSO_4$ may involve interference with ferritin, the iron-storage protein which has been shown also to have an antidiuretic action (20). (This latter possibility is rendered somewhat less likely by the finding that apoferritin, the iron-free portion of ferritin, also is antidiuretic (20)). Other ways in which $MnSO_4$ might have produced the thirst and diuresis are conceivable and are not ruled out. Whether the increased intake of water may have caused the diuresis or vice versa is not known.

The depigmentation of the incisor teeth we observed in manganese-poisoned rats may have resulted directly from lowered iron. Since rats given an excess of manganese have lower concentrations of plasma iron and lower stores of total body iron (17) and since it is known that simple iron deficiency causes loss of the yellow iron-containing pigment (21). Or, the manganese-produced depigmentation may have been secondary to a conditioned deficiency of phosphorus, since we found (herein) low values for serum phosphorus and since a diet low in phosphorus produced some bleaching of the yellow pigment (22). Hypoplasia of the enamel of the rat's upper incisor has been noted histologically after subcutaneous injection of manganese chloride, but it was not mentioned whether grossly apparent depigmentation, as seen in our manganese-fed rats, occurred also (23).

The present observations and some other work with several species indicate that an excess of manganese may notably affect the levels of hemoglobin and numbers of erythrocytes. Some of this work has been reviewed in various places (see, for example, 24). A decrease in hemoglobin and an increase in the red blood cell count, as reported in the present work, have been found in various species, although other effects or no changes also have been found. One per cent of manganese added to a stock diet for normal rats decreased hemoglobin but had no effect on the number of red cells (25). A combination of excessive oral and injected manganese in rats decreased hemoglobin (17) and, from the data presented, appeared to increase the numbers of red blood cells somewhat, although the increase in red cells is not indicated as being significant (17). Thus in the rat, lowering of hemoglobin by excessive manganese seems to be better established than is an increase in red blood cells. Also, excessive manganese promoted reticulocytosis in rats (17). Manganese in excess markedly affects both the iron picture (17, 26) and the copper picture (17, 26) in the body. However, whether manganese may produce or prolong anemia primarily by antagonism of iron or of something else (e.g. copper) is not known. Iron might appear to be involved mainly, since anemia was reversed by iron in manganese-fed pigs (27) but not by copper in rats given manganese (17).

Alkaline phosphatase of serum. Magnesium activates the alkaline phosphatase of rat serum (28, 29). The activity of this enzyme in rat serum is decreased when magnesium is deficient (30) and is either increased (31) or unchanged (32) when manganese is deficient in the diet. Thus it was of interest to learn the effects of dietary excesses of these metals on the activity, especially in view of the depression of serum magnesium by an excess of manganese in several mammalian species (18, 19), and also in view of the knowledge that magnesium and manganese may be at least partially interchangeable *in vitro* as activators of mammalian tissue alkaline phosphatases (33) and other enzymes. Our finding that the activity of serum alkaline phosphatase in the $MnSO_4$ group did not differ significantly from that in the cornstarch group may indicate that manganese was not specifically interfering with magnesium in our experiment, at least at the time we tested the enzymatic activity. However, it might also be that manganese did disturb the metabolism of magnesium in one or more other ways, but that manganese either did not displace magnesium from this particular enzyme or else was able to replace magnesium functionally as the activator of this enzyme *in vivo*. The slight but significant increase in the activity in the $MgSO_4$ group may indicate that the circulating enzyme(s) was not fully activated by magnesium under the conditions of the other diets. (Rosenthal *et al.* (29) found that plasmatic alkaline phosphatase of the rat was only about 75% as active in the absence as in the presence of added magnesium in the assay system, in a variety of nutritional states. We did not add any magnesium to our assay system.) It also seems possible that the extra magnesium of the $MgSO_4$ diet induced the formation and release (or simply the release) into the serum of more alkaline phosphatase, but we have no data on this point.

Gastrointestinal tract. An increase in the amount of manganese, added as manganese sulfate, from 2.6 ppm to about 50 ppm in the diet in *experiment a* had no effect on the weight of the cecum or small gut in our rats. This contrasts with the finding that the ceca and small gut of the chick grow heavier when the diet contains 50 ppm manganese as compared to 10 ppm manganese (3). The very much larger amounts of manganese in the high-manganese diet of our *experiment b* produced only a slight (statistically nonsignificant at the 5% level)

increase in cecal weight. Thus, it appears that the rat's cecum and small gut are either much less susceptible than the chick's to stimulation by dietary manganese, or else that other conditions in our experiments were too different from those in the experiment with chicks to be able to demonstrate a similar effect. The toxicity of manganese, particularly as reflected in poor growth, in *experiment c* prevents reaching any conclusion about effects on the rat's gastrointestinal tract of still higher levels of manganese.

Diarrhea and cecal distention, such as we noted herein with excessive $MgSO_4$ in *experiment c*, occur also with excessive dietary $MgCl_2$ or $MgCO_3$ (9). The weight of the cecum plus its contents was greatly increased by $MgCl_2$ or $MgCO_3$ (9); however, the weight of the cecal tissue alone was not given (9). Our findings with $MgSO_4$ show that the distention is accompanied by an actual increase in cecal tissue, not just by a stretching of the wall.

Many factors need to be considered before finally judging the possible usefulness of the hydragogue hypothesis to explain sulfate-produced increases in growth of any gastrointestinal region. Among these factors are the calculated numbers of extra particles per 100 gm feed which are supplied by the extra sulfate salts. These numbers for *experiment c*, assuming 100% ionization in the digestive tract, are 0.166×10^{23} for $MnSO_4$ and for $MgSO_4$, and 0.249×10^{23} for Na_2SO_4. (For comparison, the numbers of sucrose or glucose particles per 100 gm feed in previous experiments (1) were: 67% sucrose diet, 1.179×10^{23}; 25% sucrose diet, 0.440×10^{23}; 67% glucose (hydrate) diet, 2.037×10^{23}; and 25% glucose (hydrate) diet, 0.760×10^{23}. In Friedman's 50% talc diet (34), feeding of which stimulates growth of the small intestine in mice, perhaps by a hydragogue action, there are 0.794×10^{23} talc molecules per 100 gm of feed; the number of particles (molecules plus ions) from this talc probably would be somewhat greater than this, depending upon the degree of ionization in the lumen, which is unknown.) In addition to the number of particles per unit weight of feed, many other factors may affect the intensity and duration of a hydragogue action. Obviously, the total amount of feed eaten, and the amount eaten at any one meal, will influence the number of extra particles present in the tract. Other factors are extent of coprophagy, rate and completeness of digestion and absorption, rate of gastric emptying and intestinal transit, rate of excretion of absorbed substances in the bile or through the intestinal wall, and disposition by microbial action. If we consider only the number of small particles furnished by the diets, the present group of exploratory experiments does not adequately test the idea that a hydragogue action of certain substances is involved in their stimulation of organal growth. To cite just one example, in the stomach immediately after eating a similar weight of different diets (when very little absorption or emptying might have occurred), the number of extra small particles furnished by the diets high in sulfate salts is considerably less than the number of sugar particles furnished by the 67% glucose and 67% sucrose diets. The latter two diets are the only ones we have found which increase the gastric weight (1). Furthermore, the number of particles from the sulfate salts is smaller also than the number of sugar particles from 25% glucose and 25% sucrose diets, which diets fail to increase the gastric weight (1). So perhaps one might hardly have expected to have found any effect of our high sulfate diets on gastric weight, if a hydragogue effect is indeed involved in stimulation of gastric growth by the diets high in sugars, assuming that other factors are equal for the sulfate and sugar diets. However, as mentioned above, we cannot assume that these other factors are equal for all substances. Thus, differences in rate of gastric emptying could quickly change the concentration of different substances, and so alter the osmotic effect. Similar considerations apply to other factors and other regions of the tract. Until the many other factors are investigated thoroughly, the usefulness in gastrointestinal physiology of the present group of exploratory experiments is twofold. First, we have learned that feeding excessive amounts of $MgSO_4$ increases the weight of cecal tissue and appears to lengthen the colon plus rectum. Second, we have obtained some idea of the levels of sulfate salts which can be fed without untoward effects. Thus, in *experiment c*, the toxic effects of manganese sulfate and the digestive disturbances due to magnesium sulfate suggest that we could not sensibly raise the dietary levels of these two salts any higher, if it were necessary to do so to more adequately test the hydragogue theory of gastrointestinal growth stimulation. However, higher levels of sodium sulfate could be tested.

We are indebted to Mrs. Doris Elliot and Miss Margaret Thornbury for help with *experiments a* and *b*, respectively, to Mr. Robert Schlitz for the blood cell counts in *experiment c*, and to Dr. M. Moinuddin for help with the assays of *experiment c*.

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AN AUTORADIOGRAPHIC STUDY OF THE INCORPORATION OF S^{35}-LABELED SODIUM SULFATE IN DIFFERENT ORGANS OF ADULT RATS AND RABBITS[1]

By

ERIK ODEBLAD and HARRY BOSTRÖM

(Received for publication March 28th, 1952)

The presence of sulfomucopolysaccharides in several mammalian organs and tissues, e.g. gastric mucosa, skin, cartilage, aorta, sclera, cornea, has been known for a long time (*Levene* 1925, *Meyer* 1945). In most of these tissues a high uptake of S^{35} has been demonstrated by injection of S^{35}-labeled sodium sulfate in different animals (*Dziewiatkowski* 1949, *Dziewiatkowski, Benesch* and *Benesch* 1949, *Layton* 1951). The major portion of the labeled sulfate in cartilage has been found to be present in chondroitin sulfate (*Dziewiatkowski* 1951, *Boström* 1952) and also in the skin af adult rats, S^{35} was found to occur in a sulfomucopolysaccharide fraction (*Boström* and *Gardell* 1952).

Contrast-autoradiographic studies of cartilage after injection of S^{35}-labeled sodium sulfate have earlier been performed by *Dziewiatkowski* (1951) and *Boström, Odeblad* and *Friberg* (1952). Also track-autoradiography has been used (*Campbell* 1951, *Campbell* and *Persson* 1951). The sulfate fixation in different tissues of the genital organs of the female rat and rabbit has been discussed in a previous paper (*Boström* and *Odeblad* 1952).

The purpose of this investigation was to study by means of contrast-autoradiography the uptake of injected S^{35}-labeled sodium sulfate in different organs of the rat and the rabbit. Organs and tissues belonging to the gastrointestinal tract and the cardiovascular system, some parenchymatous organs, the eye and the trachea were studied. In most of the tissues where sulfomucopolysaccharides are known to be present a considerable uptake of S^{35} was demonstrated.

[1] This investigation has been aided by grants from the Therese and Johan Andersson Foundation, the Knut and Alice Wallenberg Foundation and the Vitrum Company, Stockholm.

TABLE I

Sulfate fixation in the gastrointestinal tract[1].

Number	Animal	Organ	Tissue	Amount of S^{35}
1	Rat	Esophagus	Superficial layer	—
			Epithelium	++++
			Lamina propria	(+)
			Muscularis mucosae	++
			Submucous layer	(+)
			Muscularis externae	++
			Tunica adventitia	+++
2	Rat	Fundus ventr.	Epithelium	+++
			Submucous layer	(+)
			Muscularis externae	++
3	Rat	Duodenum	Epithelium	+++
			Submucous layer	+
			Muscularis externae	++
4	Rat	Ileum	Epithelium	++++
			Submucous layer	+
			Muscularis externae	++

[1] In the tables, — denotes no S^{35}, +, ++, +++ and ++++ denote small, moderate, large and very large amounts of S^{35}.

TABLE II

Sulfate fixation in cardiovascular tract.

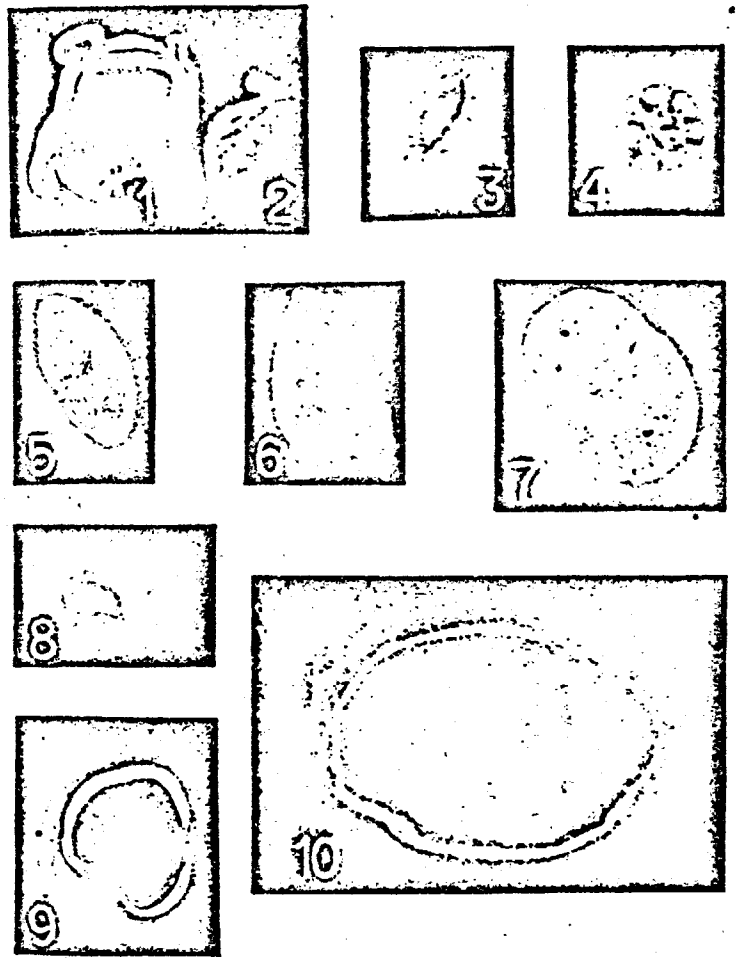
Number	Animal	Organ	Tissue	Amount of S^{35}	Notes
5	Rat	Heart	Musculature	+	Diffuse uptake
6	Rabbit	Aorta	Tunica intima	++(+)	
			Tunica media	+++	
			Tunica adventitia	++	
7	Rat Rabbit	Spleen	—	++	Diffuse uptake. A few colloidal particles are to be seen.

THE EXPERIMENT

Five adult rats weighing about 200 g and one adult rabbit were used. The rats were given 100 μC S^{35} as "carrier-free" Na_2SO_4[1] diluted with 0.1 mg non-labeled Na_2SO_4 in 0.2 ml of distilled water subcutaneously per 100 g of body weight and were killed 48 hours later. The rabbit was injected with 2.0 mC S^{35} as Na_2SO_4 in 4 ml of distilled water, containing 0.2 mg carrier, and was killed 48 hours after the injection.

Various organs were removed immediately after death and fixed in methanol for 24 hours. They were then treated in propanol for 3 hours, embedded in paraffin and cut into a thickness of 10 μ. The

[1] Supplied by A.E.R.E., Harwell, England.



Figs. 1–10.

Contrast-autoradiographs of different organs of the adult rat after intraperitoneal injection of 100 μC per 100 g of body weight of S^{35}-labeled sodium sulfate (× 10).

1. Stomach 2. Esophagus 3. Duodenum 4. Ileum 5. Spleen 6. Liver 7. Kidney 8. Aorta 9. Trachea 10. Eye (cornea below)

TABLE III

Sulfate fixation in some parenchymatous organs.

Number	Animal	Organ	Tissue	Amount of S^{35}	Notes
8	Rabbit Rat	Liver	—	+(+)	Diffuse uptake. A few colloidal particles are to be seen.
9	Rat	Kidney	Cortical substance Medullary substance	++(+) +(+)	Most of S^{35} was retained in the convoluted tubules in the cortical zone. In the glomerular and capsular epithelium of the glomerulus there was a medium uptake.
10	Rabbit	Lung	Respiratory epithelium Cartilage plates Lymphatic follicles	+(+) +++(+) (+)	The alveolar structure of the lung was seen.
11	Rabbit	Pancreas		++	Diffuse uptake. Higher uptake in the pancreatic acini than in the islands of Langerhans.
12	Rabbit	Thyroid gland	—	++	Diffuse uptake. Higher uptake in epithelial cells than in the lumen of the follicles.

TABLE IV

Sulfate fixation in eye and trachea.

Number	Animal	Organ	Tissue	Amount of S^{35}	Notes
13	Rat	Eye	Cornea Sclera Vitreous body	++++ +++ —	In cornea most of S^{35} was retained in the inner part.
14	Rat Rabbit	Trachea	Cartilage Epithelium	++++ +	

sections were mounted on methacrylate slides. They were then deparaffinated in xylene and in ethanol and pressed against Gevaert Dentus Rapid film in iron presses for contact autoradiography. The time of exposure was 33 days. Development was undertaken with Kodak DK 20 with potassium bromide added to 0.2 per cent. The time of development was 30 minutes at 18.3° C. After fixing, washing and drying, the reverse side was removed with Farmer's solution in excess and the films were again washed and dried. They were then ready for analysis. The resolution of the autoradiographs appeared to be about 20 μ. A total of 120 autoradiographs were analysed.

On analysis, the autoradiographs were compared in the microscope with haemalum-eosin stained sections. A quantitative judgement of the exposure was justified, as the development was controlled by a standardized light exposure on each piece of film, and special experiments indicated that the density was directly proportional to the exposure.

RESULTS AND DISCUSSION

The results are given in Tables I to IV and are exemplified in Figs. 1 to 10.

The histologic treatment used causes a retention in the sections not only of free and ester-bound sulfates but also of other sulfur-containing compounds such as methionine, cysteine and taurin. In the last-mentioned compounds, however, an extremely low, or no significant, uptake of S^{35} takes place when the isotope is given as sulfate (*Tarver* and *Smith* 1939). Therefore free and ester-bound sulfur may be considered to be the main labeled components in the tissues investigated. From the autoradiographs only it is not possible to decide whether S^{35} occurs as free or ester-bound sulfates. Certain facts, however, speak in favour of an incorporation of the sulfate administered as ester-bound sulfates in different kinds of mucopolysaccharides. Such an incorporation was demonstrated in chondroitin sulfuric acid prepared from cartilage. (*Dziewiatkowski* 1951, *Boström* 1952) and in a sulfomucopolysaccharide fraction prepared from the skin of rats (*Boström* and *Gardell* 1952). In the present investigation the highest uptake of

the S^{35}-labeled sulfate was found in those tissues where sulfomucopolysaccharides are known to be present. Finally, free sulfates injected in rats are known to be excreted very rapidly (*Dziewiatkowski* 1945).

SUMMARY

The uptake of injected S^{35}-labeled sodium sulfate by different organs of the rat and rabbit has been studied by means of contrast-autoradiography. In most of the tissues where sulfomucopolysaccharides are known to be present a considerable uptake of S^{35} was demonstrated.

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Corps étranger dans le palais du Cheval

par le dr J. Macrodès
vétérinaire à Ghida (Salonique)

La présence de corps étranger dans la bouche des Equidés n'est pas souvent citée dans la littérature. C'est pour cela que nous croyons intéressant de relater un accident que nous avons rencontré trois fois dans l'espace de 8 ans.

Il s'agit du corps étranger du palais; une tige rigide qui se trouve coincée entre les arcades molaires supérieures à la hauteur de la deuxième ou la troisième dent et qui dépasse en épaisseur quelque peu le niveau des tables. Ce corps, en comprimant la langue pendant la fermeture de la bouche, lui interdit tout mouvement de sorte que la mastication et la déglutition sont fortement gênées et les aliments sont rejetés par la bouche avec de la salive.

Dans deux de nos cas, le corps étranger était un épi de maïs jeune et tendre, vide de grains; dans le troisième, il s'agissait d'un morceau de bois.

Ces corps étrangers dépassaient, d'un demi-centimètre à peu près, le niveau des tables dentaires et ils étaient râpés en surface à leurs extrémités par le mouvement horizontal des molaires inférieures.

A l'inspection de la bouche, le corps était dissimulé dans un amas d'herbes. Dans l'un des cas, le cheval souffrant depuis deux semaines nous était présenté, après avoir subi tous les remèdes empiriques du village, dans un état de cachexie extrême.

La bouche ouverte au pas d'âne, un simple coup de doigt suffit pour extraire le corps et rétablir la préhension régulière des aliments.

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127: 287-95 (1951)
382

Purpura hémorragique des Bovidés, consécutif à l'ingestion de sulfate d'ammoniaque

par M. Priouzeau
vétérinaire à Mareuil-sur-Lay (Vendée)

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Les causes du purpura hémorragique chez les Bovidés sont rarement bien connues. Cependant il nous a été donné d'observer cette affection dans des conditions étiologiques parfaitement précises (ingestion de sulfate d'ammoniaque) et c'est pour cela que nous pensons qu'il y a un certain intérêt à décrire les symptômes observés sur les animaux vivants et les lésions relevées sur les cadavres.

Dans le but de préciser l'action exacte du sulfate d'ammoniaque sur l'organisme des Bovidés, nous mentionnerons également le résultat de nos recherches expérimentales faites à partir de ce sel employé comme engrais (*).

***

Nous sommes appelé le 21 mars 1934 chez M. C..., à T.... dans le but d'examiner les deux vaches qu'il possède et qui ont présenté, dans la nuit du 20 au 21 mars, des coliques assez violentes.

Lors de notre visite, à 11 heures, nous trouvons :

1° le cadavre encore chaud d'une vache parthenaise âgée de 3 ans. L'animal, en très bon état d'entretien, a présenté, dans la nuit, des coliques violentes avec rejet d'excréments sanguinolents et — aux dires du propriétaire — « est mort dans des conditions tragiques »;

2° une vache parthenaise âgée de 7 ans. L'animal, bien portant dans la journée du 20 mars, a absorbé, au repas du soir, une forte ration de colza. Dans la nuit, il a présenté, ainsi que son congénère soumis à la même alimentation, de violentes coliques qui persistaient encore lors de notre visite.

Son examen, à 11 heures du matin, soit 15 heures environ après le repas du soir, nous met en présence de l'un des tableaux cliniques les plus impressionnants qu'il nous ait été donné d'observer au cours de notre carrière.

L'animal est debout, trépignant, en proie à des tremblements extrêmement violents. Le faciès est crispé; les oreilles sont

(*) M. Priouzeau : Extrait d'un mémoire présenté à l'Académie vétérinaire, en 1944.

droites et raides; la bouche est légèrement ouverte; les naseaux sont dilatés, les yeux, exorbités et luisants avec des conjonctives œdématiées et brunâtres; la sclérotique, légèrement blanchâtre, est parsemée d'un piqueté hémorragique bleuâtre. Le cœur bat violemment et ses bruits s'entendent à plusieurs mètres de distance; un pouls veineux extrêmement fort parcourt avec une grande rapidité les jugulaires notablement augmentées de volume et tendues.

L'essoufflement est considérable et est accompagné de mouvements de la cage thoracique d'une ampleur extraordinaire. Le ventre, extrêmement levretté, suit la cage thoracique dans ses mouvements désordonnés. La queue, légèrement surélevée, laisse apparaître un anus béant par lequel s'échappent des excréments diarrhéiques, sanguinolents, mousseux et bulleux; excréments qui souillent la pointe des jarrets et le périnée.

La peau, surtout au niveau des mamelles, du plat des cuisses et de la vulve est recouverte d'une multitude de taches bleues ou violacées, dont la dimension va de celle d'un grain de mil à celle d'un grain de blé et qui conservent, à la pression des doigts, leur forme, leur étendue et leur coloration; il s'agit donc de pétéchies.

Parfois, le malade chancelle et semble devoir tomber; il n'en est rien et, rapidement, il reprend son aplomb. D'autres fois, au contraire, il tombe sur le sol comme une masse inerte tout en poussant des beuglements sinistres que l'on entend à plusieurs centaines de mètres de distance; il se raidit alors violemment et la mort semble devoir être proche; il n'en est rien et, grâce à des prodiges d'énergie, il se relève encore une fois.

Puis, brusquement, le moribond retombe, agite l'air de ses quatre membres, ouvre légèrement la bouche pendant que l'œil pirouette dans l'orbite et qu'un mince filet d'urine s'écoule de la vulve violacée; il pousse encore quelques beuglements et la mort fait son œuvre au cours de quelques courtes convulsions.

Le diagnostic est relativement facile.

L'angoisse extrême du malade caractérisée, par les beuglements sinistres et la violence des troubles respiratoires et cardiaques; l'évacuation d'excréments diarrhéiques, bulleux, rougeâtres et même noirâtres avec apparition concomitante d'innombrables pétéchies sur certaines parties de la peau et sur la sclérotique, sont autant de signes qui permettent d'affirmer que l'on se trouve en présence de purpura hémorragique.

Aux dires du propriétaire, le premier animal atteint avait présenté des symptômes identiques. Ce fait nous permettait de penser à la nature toxi-alimentaire de la maladie.

Nous procédons sur le champ à l'autopsie des deux cadavres.

La peau et le tissu conjonctif sous-cutané présentent en abondance des pétéchies.

Les muscles, un peu décolorés, sont recouverts, en de nombreux points de leur surface et plus particulièrement au niveau des aponévroses, de très nombreuses pétéchies de coloration bleuâtre ou violacée. Dans la masse musculaire même, on rencontre d'assez nombreux petits caillots sanguins brunâtres.

L'absence totale de lésions sur les muqueuses buccale et œsophagienne contraste fortement avec l'importance des lésions observées sur le reste du tractus digestif.

Les séreuses de l'estomac et de l'intestin sont, à leur surface et en profondeur, le siège de vastes taches hémorragiques de coloration rouge, bleuâtre ou violacée; taches qui sont, soit isolées, soit confluentes et forment alors soit d'immenses nappes sur le rumen, le réseau et surtout la caillette, soit de vastes manchons surtout développés au niveau du grêle.

La muqueuse de cet intestin et celle du cæcum sont rouge violacé, fortement œdématiées en certains points, ulcérées, hémorragiques en d'autres points.

La caillette, normalement vide de corps étrangers, contient environ 300 grammes de sable fin identique à celui que l'on rencontre si souvent dans le réseau; sa muqueuse est fortement œdématiée, rougeâtre et parsemée de taches ecchymotiques gagnant la profondeur vers la séreuse.

Le réseau, complètement vide d'aliments et de corps étrangers, a ses alvéoles recouvertes d'un liquide légèrement bouillonnant et devenant un peu lactescent au contact de l'air. Il semble bien que cette vidange totale du réseau avec passage de son contenu dans la caillette soit l'indice de l'existence de violentes contractions au niveau de cet organe au cours de la maladie; contractions ayant abouti à un véritable balayage de la muqueuse. La muqueuse du rumen est légèrement congestionnée.

Toute la portion aponévrotique de la face postérieure du diaphragme est recouverte d'une vaste nappe hémorragique de coloration violacée.

Le foie, notablement augmenté de volume, est décoloré, jaune pâle et très friable.

Le poumon, quasi exsangue, est blanc nacré et parsemé de

quelques pétéchies sur sa plèvre. Le myocarde est également décoloré, de couleur feuille morte et recouvert d'innombrables pétéchies surtout au niveau de la graisse du sillon auriculo-ventriculaire. L'endocarde ne présente que peu de pétéchies.

Sur cet animal, âgé de 3 ans, le thymus n'est plus qu'un véritable amas de pétéchies bleues ou violacées.

Le sang n'est pas coagulé; il est fluide, faiblement rouge et tache à peine les doigts.

Le sable de la caillette et le liquide lactescent du réseau furent prélevés et, grâce à l'obligeance de notre ami, M. G..., pharmacien à T..., nous pûmes mettre en évidence la présence du sulfate d'ammoniaque.

Connaissant la cause de la mort des animaux, il nous paraissait intéressant de rechercher le mécanisme de l'absorption de ce produit toxique.

Une visite complète de l'étable permit d'en préciser la nature.

Deux sacs de sulfate d'ammoniaque avaient été placés sur une planche, fixée dans le mur à un mètre au-dessus du sol. Au-dessous de cette planche, le fourrage — en l'occurrence du colza — était amoncelé et distribué aux animaux, le plus souvent après avoir séjourné douze à dix-huit heures dans cet emplacement. Or, le sulfate d'ammoniaque est un produit très déliquescent et il nous fut facile de constater que de très nombreuses gouttelettes tombaient des sacs et arrosaient lentement le fourrage placé en dessous. Cet aliment, ainsi recouvert de ce produit toxique, était ingéré par les animaux avec d'autant plus d'avidité que le colza était très appétissant et le sulfate d'ammoniaque inodore et peu caustique. Ce sel séjournait d'abord dans l'estomac où il agissait comme un véritable purgatif (balayage du réseau), puis il se rendait rapidement dans l'intestin où son séjour prolongé et son absorption permettaient le déclenchement des troubles morbides et des lésions de purpura hémorragique.

*
* *

Il est facile de concevoir l'intérêt qu'il y aurait à rechercher :

1° A quelle dose le sulfate d'ammoniaque, utilisé comme engrais, est toxique pour les Bovidés;

2° Quelle est l'action du sulfate d'ammoniaque sur le sang *in vitro* et *in vivo*?

3° Si le sulfate d'ammoniaque, mélangé à des aliments, puis ingéré lentement, est plus toxique qu'en ingestion massive en solution aqueuse?

4° Si, sous forme d'engrais, répandu sur le sol, puis assimilé par les plantes, le sulfate d'ammoniaque peut, par leur intermédiaire, favoriser l'installation d'un état d'hémophilie passagère puis celle du purpura hémorragique ?

5° Enfin, puisque, avec ce sel, il est possible de produire le purpura hémorragique s'il y aurait un moyen de pouvoir rechercher le mécanisme intime de la production des hémorragies qui apparaissent au cours de cette intoxication.

Dans le but de rechercher cette toxicité, nous avons fait ingérer à des bovins d'expérience des solutions concentrées de sulfate d'ammoniaque.

Première expérience

Une vache, âgée de 6 ans, en moyen état d'entretien et pesant 350 kilos environ, reçoit, à la bouteille, le soir à 18 heures, un breuvage contenant :

Sulfate d'ammoniaque du commerce	150 grammes
Eau ordinaire Q. S.	1 litre

Au cours de la nuit, l'animal présente des coliques assez violentes, mais n'offre point les troubles morbides aussi alarmants que ceux observés cliniquement. Le matin à 7 heures — soit douze heures environ après l'ingestion du produit toxique — il mourait. Une diarrhée abondante et sanguinolente s'était installée comme le prouvaient les excréments souillant l'arrière-train du malade et le sol de l'étable. Des pétéchies rouges ou violacées recouvraient la peau du plat des cuisses et de la mamelle; on en voyait également sur les conjonctives et surtout sur la sclérotique.

Le sang était extrêmement fluide, tachant à peine les doigts et ne coagulant pas ou coagulant tardivement à l'air.

On ne relevait pas trace de la moindre lésion sur la bouche et l'œsophage.

Dans la cavité thoracique et dans la cavité abdominale, on observait un ensemble de lésions tout à fait comparables à celles signalées sur les deux vaches.

Deuxième expérience

Un bouvillon de quinze mois, maigre et pesant 140 kilos reçoit en breuvage à la bouteille :

Sulfate d'ammoniaque du commerce	40 grammes
Eau ordinaire	500 centimètres cubes

La mort survient au bout de dix heures. L'animal a présenté des coliques assez graves et des troubles morbides identiques à ceux constatés sur la vache de M. C...; troubles cependant beaucoup moins violents.

Les lésions observées tant sur la peau que sur les viscères, sont absolument identiques à celles précédemment décrites.

De ces deux expériences, il résulte que :

a) Le sulfate d'ammoniaque du commerce en solution aqueuse au 1/10 et à la dose maxima de 1 gramme de sel par deux kilos de poids vif, détermine, par ingestion, la mort des Bovidés en douze à quinze heures;

b) Les troubles morbides et les lésions observées ne peuvent se rapporter qu'au purpura hémorragique;

c) Les troubles morbides produits au cours de l'ingestion du sulfate d'ammoniaque en breuvage sont moins bruyants que ceux observés au cours de l'ingestion de ce produit avec les aliments. Peut-être l'assimilation de ce sel est-elle meilleure dans ce dernier cas?

Action du sulfate d'ammoniaque sur le sang de bovin *in vitro*

Ce sel s'est montré — en ingestion — toxique et mortel à la dose de 150 grammes chez un bovin pesant 350 kilos et pouvant avoir environ 10 litres de sang.

En outre, ce sel avait déterminé non seulement le purpura hémorragique, mais encore un état spécial du sang (retard à la coagulation) qui ne pouvait se rapporter qu'à l'hémophilie.

En un mot, 150 grammes de sel d'ammoniaque avait empêché la coagulation de 10 litres de sang. Nous avons donc préparé une solution contenant :

Eau distillée Q. S.	0 gramme 15
Eau distillée Q. S.	1 centimètre cube

et nous avons procédé comme suit :

TUBES A ESSAI ORDINAIRES contenant chacun 10 cc. de sang frais recueilli à la veine jugulaire	DOSE DE LA SOLUTION S.	TEMPS POUR OBTENIR la coagulation
I	sans solution	8' à 10'
II	1/4 cc.	9' à 10'
III	1/2 cc.	9' à 10'
IV	1 cc.	13' à 20'
V	1 cc. 1/2	plusieurs jours

Or, 1 cc. et demi de solution contenait environ 0 gr. 25 de sulfate d'ammoniaque.

Il s'ensuit donc que 25 grammes de sulfate d'ammoniaque dissous dans 150 cc. d'eau, peuvent empêcher la coagulation d'un litre de sang. On doit pouvoir réduire la quantité d'eau et avec une solution de :

Sulfate d'ammoniaque	25 grammes
H^2O	50 grammes

nous avons obtenu la non-coagulation du sang pendant plusieurs jours.

Action du sulfate d'ammoniaque sur le Lapin

Le lapin étant un herbivore, il était logique de rechercher l'action du sulfate d'ammoniaque sur son organisme.

Nous avons donc fait la série d'expériences ci-après :

Expérience I. — Un lapin de 3 kilos, en excellente santé, reçoit, à 9 heures, en injection sous-cutanée, 2 grammes de sulfate d'ammoniaque dissous dans 5 cc. d'eau;

De 9 heures à 9 heures 15 : essoufflement progressif;

A 9 h. 15, l'animal se met en décubitus latéral complet et reste très étouffé.

A 9 h. 25, on compte cent respirations par minute. Le cœur bat assez vite et violemment.

De temps en temps, l'animal est secoué par de brusques convulsions. On entend des borborygmes assez intenses.

A 9 h. 30, l'animal commence à respirer par la bouche. On note trois légers soulèvements consécutifs des côtes, puis brusquement la bouche s'ouvre comme pour aspirer de l'air et, en même temps, il se produit un mouvement très ample de la cage thoracique. On note vingt-cinq ouvertures de la bouche par minute.

Au cours de la période de 9 h. 30 à 9 h. 50, l'animal présente trois violentes crises tétaniformes. Brusquement, il se raidit, allonge fortement ses quatre membres, rejette la tête en arrière et contracte violemment tous les muscles de l'économie; en même temps, on entend des borborygmes intenses.

Les crises durent 30 secondes environ (coliques).

A 9 h. 50, les mouvements des côtes sont inexistants et on ne compte plus que 10 ouvertures de la bouche par minute.

A 10 h. 15, il n'existe plus que 5 ouvertures de la bouche par minute et l'animal semble devoir mourir bientôt.

Il n'en est rien; les mouvements d'ouverture de la bouche deviennent de moins en moins amples pour devenir imperceptibles et la mort ne se produit qu'à 13 h. 30, dans le calme.

A noter que de 9 h. 30 à 13 heures, on observe au niveau des jugulaires du pouls veineux; le nombre des pulsations atteignant par moment 130 à la minute. Le réflexe oculaire était nul.

A 9 h. 30, l'extrémité de la veine de l'oreille est sectionnée et un sang rouge, fluide, s'écoule avec une extrême rapidité. Il est indispensable de ligaturer l'extrémité de l'oreille pour éviter une hémorragie grave.

Une goutte de sang est placée sur une lame de verre séchée.

La goutte reste déformable jusqu'à 10 h. 45; le retard à la coagulation a donc été de plus d'une heure.

Dès la mort, nous pratiquons l'autopsie.

Le sang recueilli sur une lame de verre et dans un tube en verre de 3 centimètres de largeur, ne coagule qu'au bout d'une heure environ.

On trouve quelques pétéchies dans le conjonctif sous-cutané situé au niveau du point d'injection de la solution; mais on ne rencontre point les lésions classiques du purpura hémorragique.

Expérience II. — Un lapin de 2 kilos en excellente santé, reçoit, sous la peau, 2 grammes de sulfate d'ammoniaque dissous dans 4 cc. d'eau : à 16 heures, injection sous-cutanée au cou.

Les troubles observés sont les mêmes que ceux rencontrés au cours de l'expérience I. Les troubles morbides sont apparus aux mêmes époques et la mort de l'animal s'est produite à 20 heures, soit quatre heures après l'injection de sulfate d'ammoniaque.

Expérience III. — Un lapin de 1 kilos, très gras, en excellente santé, reçoit une injection sous-cutanée de 2 grammes de sulfate d'ammoniaque dissous dans 5 cc. d'eau.

A 16 heures, injection sous-cutanée. Le sang, avant l'injection, coagule sur une plaque de verre en 10 minutes.

A 16 h. 10, forte accélération de la respiration; battements cardiaques brusques et violents.

A 16 h. 15, l'animal se met en décubitus latéral complet et respire plus lentement.

A 16 h. 20, le cœur bat encore régulièrement, mais avec violence; un superbe pouls veineux se voit au niveau des jugulaires.

A 16 h. 40, les mouvements de la cage thoracique deviennent quasi imperceptibles; 5 ou 6 par minute. Le lapin ouvre fortement la bouche, soulève fortement ses côtes comme pour aspirer de l'air. Nous pensons que l'animal va mourir; le réflexe oculaire est nul. Ce mode respiratoire persiste jusqu'à 16 h. 45.

A 16 h. 45, progressivement la bouche s'ouvre. 8, 10, 15 fois par minute et en même temps les mouvements de la cage thoracique deviennent quasi normaux; mais l'animal respire toujours par la bouche.

A 17 h. 05, les naseaux commencent à s'ouvrir légèrement, la bouche se referme progressivement et le jeu des côtes devient quasi normal à 17 h. 20.

A 17 h. 20, la respiration devient quasi normale, quoique notablement agitée. L'animal commence à donner signe de vie, il agite un peu les oreilles et le réflexe oculaire réapparaît.

A 18 heures, tout est rentré dans l'ordre et l'animal se déplace normalement.

A noter que de 16 h. 20 à 17 heures, on entendait souvent de violents borborygmes, mais l'animal n'a pas présenté de crise tétaniforme grave.

Le sang provenant de la section de la veine marginale de l'oreille est rosé, fluide et placé sur une lame de verre, il ne coagule qu'au bout d'une heure environ. On ligature l'oreille pour éviter l'hémorragie.

L'animal a été conservé quatre jours, puis consommé sans le moindre inconvénient. A cette époque, le sang a coagulé environ 10 minutes après avoir été placé sur une lame de verre.

Expérience IV. — Un lapin de 2 kgs 500, gras, en excellente santé, reçoit 1 gramme de sulfate d'ammoniaque dissous dans 4 cc. deau (injection sous-cutanée).

A 11 h. 20, injection sous-cutanée.

A 11 h. 30, essoufflement notable.

A 11 h. 40, cœur violent et agité, 110 mouvements respiratoires à la minute.

A 11 h. 55, l'animal respire moins vite et se met en décubitus latéral complet. On note 40 mouvements respiratoires par minute.

A 12 h. 05, violente crise tétaniforme avec borborygmes assez intenses.

A 12 h. 15, l'animal commence à respirer par la bouche et les mouvements de la cage thoracique sont très faibles.

Le réflexe oculaire existe. Il y a un pouls veineux très net au niveau des jugulaires.

A 12 h. 40, la respiration se fait à nouveau par le nez, les mouvements de la cage thoracique redeviennent plus amples et l'animal soulève la tête.

A 13 heures, l'animal reprend une position normale et cherche à se déplacer.

A 13 h. 15, tout est rentré dans l'ordre.

A 13 h. 20, le sang provenant de la veine marginale de l'oreille est rosé, fluide et ne coagule que 50 minutes après avoir été déposé sur une lame de verre. On doit ligaturer l'oreille pour éviter une hémorragie grave.

De ces expériences, il résulte que le lapin est très sensible à l'action du sulfate d'ammoniaque en injection sous-cutanée.

Dès que la dose dépasse 50 centigrammes par kilo de poids vif, elle devient mortelle pour cet animal.

Si la dose est inférieure à 50 centigrammes, on détermine des troubles graves, mais la santé renaît très rapidement.

Dans tous les cas, on détermine chez l'animal, non seulement des troubles généraux graves, mais un état d'hémophilie passagère caractéristique.

Nous pensons devoir signaler que le sulfate d'ammoniaque, utilisé au cours de ces expériences, était le produit livré au commerce comme engrais. Avec un produit chimiquement pur, les doses pourront être légèrement différentes.

TABLE 5.—*Effect of phosphorus level, type of carbohydrate, and amino acid composition of the diet on reproductive performance, feed consumption, and body weight gain of Japanese quail*

Diet[1]	Dietary variation	Egg prod. (%)	Egg wt. (g.)	% Hatch incubated eggs	Feed/quail day (g.)	Av. body wt. gain (g.)
A		88.5	10.15a[2]	75.8a	27.1	15.0
B	1.24% P	73.6	9.11b	52.1c	23.5	2.7
D	1.00% P	73.8	8.89b	55.9bc	23.2	3.3
E	Corn starch	80.5	9.26b	69.1a	25.6	2.2
F	Amino acids	74.9	9.14b	65.5ab	23.3	5.6

[1] See Table 1 for composition of diets.
[2] Means not having the same letter are significantly different at the 5% probability level.

ental diets, the quail were fed a puri-
diet (Diet D, Table 1) for ten days.
were collected on days 8, 9, and 10.
eggs from each pen were combined for
hree days and an average egg weight
mined. This value was designated the
reatment average. The quail were then
the experimental diets for ten days.
were again collected on days 8, 9, and
and an average egg weight calculated
each pen. This weight was designated
reatment average. The percent change
g weight for each pen was determined
llows:

$$\frac{\text{Treatment average} - \text{Pre-treatment average}}{\text{Pre-treatment average}} \times 100 = \%\ \text{change}$$

description of treatments is listed in
le 6. The ash was prepared by heating

TABLE 6.—*Ten day assay for effect of practical diet, purified diet, or purified diet containing ash of practical diet upon Japanese quail egg weight*

Type diet[1]	% Increase in egg weight
Practical (A)	12.0a[2]
Purified D	1.2b
Purified D + ash[3]	0.4b

[1] See Table 1 for composition of diets.
[2] Means not having the same letter are significantly different at the 5% probability level.
[3] Administered as ash from an equivalent amount of practical diet.

the practical diet at 550° F. for eight hours in a muffle furnace. Three pens of five females per pen were assigned each treatment.

Results and Discussion. The results of the ten-day assay showed a significant response when the quail were changed from the purified diet to the practical diet (Table 6). When the ash of the practical diet supplied the minerals in the purified diet, no change in egg weight was observed. These data indicate that the component of the practical diet which causes increased egg size is not inorganic.

SUMMARY

Japanese quail fed a nutritionally adequate purified diet (within the limitations of our present knowledge) had a lower rate of egg production, egg weight, and hatchability than quail fed a practical diet.

Two percent safflower oil adequately supplied the essential fatty acids for reproduction in quail and added fiber elicited no reproductive response. Egg size depression is evidently not due to an essential fatty acid deficiency, protein deficiency, or amino acid imbalance or deficiency in the purified diet used.

A ten-day assay for egg weight increase is described. The egg weight increase noted when Japanese quail are changed from a purified to a practical diet is apparently

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not due to an inorganic component as the ash of the practical diet added to the purified diet failed to improve performance of the quail.

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The Response of Chicks to Sodium Sulfate Supplementation of a Corn-Soy Diet[1]

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INORGANIC sulfate has been shown to be incorporated into chondroitin sulfate (Layton, 1951), heparin (Eiber and Danishefsky, 1957), eggs (Machlin *et al.*, 1953), feathers, gizzard lining, liver, and muscle (Machlin *et al.*, 1954), taurine (Martin *et al.*, 1966; Miraglia *et al.*, 1966), and bile (Martin and Patrick, 1966). In spite of these extensive investigations into the metabolism of inorganic sulfate in animal tissues, little effort has been directed toward determining whether a dietary requirement exists for inorganic sulfur. The generally accepted precept is that the total sulfur requirement of most animals may be satisfied by the sulfur amino acids, and that any requirement for inorganic sulfur may be met by the oxidation of these amino acids.

The chickens' need for inorganic sulfur was demonstrated by Machlin (1955) and Gordon and Sizer (1955) when they obtained growth responses from the addition of 0.5 percent of Na_2SO_4 to a low-cystine, sulfate-free, casein-gelatin synthetic diet. However, the literature beyond this point appears devoid of further work on the subject, although Michels and Smith (1965), on the basis of work done with rats, suggested that there is a need for inorganic sulfur in the diet to prevent an increase in the sulfur amino acid requirement above that normally stated.

In a recent study using a purified diet, Ross and Harms (1970) confirmed the response of the chick to inorganic sulfate in the presence of 0.83 percent sulfur amino acids with broiler chicks and 1.23 percent sulfur amino acids with S.C.W.L. cockerel chicks. A consistent response was obtained with levels of Na_2SO_4 between 0.08 and

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TABLE 1.—*Composition of basal diet*

Ingredients	%
Yellow corn, ground	58.0
Soybean meal (50% protein)	30.6
Tallow	6.0
Alfalfa, dehy. (20% protein)	2.5
Limestone, ground	0.9
Defluorinated phosphate	1.1
Salt, iodized	0.4
Micro-ingredient mix[1]	0.45
Zoamix[2]	0.05

[1] The micro ingredient mix supplied the following per kg. of finished feed: $MnCO_3$, 83.6 mg.; vitamin A, 6000 I.U.; vitamin D_3, 2200 I.C.U.; menadione dimethylpyrimidinol bisulfite, 2.2 mg.; riboflavin, 4.4 mg.; pantothenic acid, 13.2 mg.; niacin, 39.6 mg.; choline, 499.4 mg.; vitamin B_{12}, 22 μg.; and ethoxyquin, 0.01275% of the diet.

[2] Trade name of Dow Chemical Co., containing 25% zoalene (3,5-dinitro o-toluamide).

0.32 percent. From the data obtained in this study it appeared that the chick either was unable to utilize the sulfur amino acids to supply its inorganic sulfur needs or was very inefficient in carrying out the conversion. These data suggested, therefore, that it would be more economical to meet the chickens' requirement for inorganic sulfur directly rather than with supplemental methionine. The present investigation was designed to determine the response of broiler chicks to forms of inorganic sulfur in a diet containing natural feedstuffs.

MATERIALS AND METHODS

Peterson × Peterson broiler chicks were used in experiments 1 and 2 while Hubbard × Shaver broiler chicks were used in experiments 3 and 4. All chicks received the basal diet shown in Table 1 with or without supplemental methionine and/or anhydrous sodium sulfate according to the experimental plan. The basal diet was designed to be low both in sulfur amino acids and in added sulfur compounds. This diet was calculated to contain 0.40 percent methionine and 0.39 percent cystine according to nutrient analyses of Maddy *et al.* (1963). The supplements were added at the expense of corn. Tap water containing 28 p.p.m. of SO_4 was provided throughout the experiments. Experiments 1, 2, and 3 were carried out to eight weeks of age in a broiler house consisting of 5 foot by 5 foot floor pens, with peanut hulls as litter. Each pen was provided with a 250 watt infrared brooding lamp, a hanging feeder and a waterer. Experiment 4 was a 17-day study carried out in heated battery brooders with raised wire floors.

One-week-old straight-run chicks were used in experiment 1. Ninety chicks were distributed at random into nine pens containing ten chicks each, and triplicate pens assigned to each treatment. The control groups received the unsupplemented basal diet, while one of the treatment groups was fed the basal diet supplemented with 0.075 percent of DL methionine, and the other treatment group was fed the basal diet supplemented with 0.5 percent of reagent grade Na_2SO_4.

In experiment 2, 960 day-old sexed chicks were distributed into 48 pens, with ten male and ten female chicks per pen. Triplicate pens were randomly assigned to each of the 16 treatments which consisted of 0, 0.05, 0.10, and 0.15 percent of supplemental methionine fed in combination with 0, 0.1, 0.3, and 0.5 percent of Na_2SO_4.

A 3 × 3 factorial arrangement was used in experiment 3 with four pens of 18 males and four pens of 18 females assigned to each of the nine treatments which consisted of combinations of 0, 0.05, and 0.1 percent of methionine and/or 0, 0.05, and 0.1 percent of Na_2SO_4.

In the final experiment, 990 day-old sexed chicks were distributed, five male and five female chicks per pen. The experimental plan followed that of experiment 3, in which triplicate pens were fed combinations of 0, 0.05, and 0.1 percent methionine and/or Na_2SO_4. In addition, equivalent levels of sulfur based on the sulfur content of $CaSO_4$, $Na_2S_2O_3 \cdot 5H_2O$, $NaHSO_3$, and $MgSO_4$ were fed in similar factorial designs.

Body weight gains and feed efficiency were analyzed for statistical significance by means of the analysis of variance (Snedecor, 1956) and by Duncan's (1955) multiple range test. In experiments 2, 3, and 4 the male and female data were pooled since there was no sex × treatment interaction.

RESULTS AND DISCUSSION

Experiment 1. The data summarized in Table 2 confirms that the basal diet was low in methionine as well as in inorganic sulfate, since a numerical increase in body weight was obtained from supplementation with 0.075 percent of methionine or 0.5 percent of Na_2SO_4. However, because of considerable variation the differences were not great enough to be statistically significant. Similar non-significant improvements were noted in feed conversion.

Experiment 2. With two exceptions the supplementation of the basal diet with 0.1 to 0.5 percent of Na_2SO_4 resulted in increased weight gains even in the presence of up to 0.15 percent of added methionine (Table 3). Only a few individual group gains were great enough to be significant; however, the weight gains of the pooled sulfate treatments were found to be significantly greater than those of the unsupplemented basal diets. There was also a general response from added increments of methionine to the basal diet. Although these weight gain increases were not great enough to be statistically significant, they were consistent enough so that the pooled data showed a difference between the upper and lower levels fed.

TABLE 2.—*Mean body weights and feed conversion of broiler chicks fed supplemental methionine or sodium sulfate in a corn-soy diet from one to eight weeks (experiment 1)*

Treatment	Body weight, g.			Feed/gain
	Male	Female	Mean	
Control	1,459	1,246	1,353	2.23
+0.075% methionine	1,542	1,303	1,423	2.18
+0.5% Na_2SO_4	1,580	1,277	1,429	2.18

TABLE 3.—*Mean body weight gains and feed conversion of broiler chicks fed graded levels of methionine and sodium sulfate to eight weeks of age (experiment 2)*

Methionine, %	Sodium sulfate in diet (%)				Mean
	0	0.1	0.3	0.5	
	Body weight gain (g.)				
0	1185	1289	1268	1196	1234[ab1]
0.05	1245	1256	1268	1236	1251[a]
0.10	1279	1385	1272	1325	1315[b]
0.15	1300	1337	1357	1311	1334[b]
Mean	1252[a]	1317[b]	1291[b]	1275[ab1]	
	Feed gain				
0	2.40	2.33	2.33	2.35	2.35[a]
0.05	2.20	2.25	2.38	2.27	2.28[b]
0.10	2.14	2.22	2.21	2.18	2.19[c]
0.15	2.19	2.21	2.19	2.22	2.20[c]
Mean	2.23[a]	2.23[a]	2.26[a]	2.26[a]	

[1] Mean values in the same column or row with different superscripts are significantly different by Duncan's multiple range test, ($P < 0.05$).

Feed conversion generally improved with increasing methionine level, but not with Na_2SO_4 supplementation (Table 3), although there was a small improvement in feed conversion at all levels of Na_2SO_4 supplementation in the basal groups fed no added methionine. These differences were of the magnitude observed in experiment 1, but again were not significant.

Experiment 3. Lower levels of Na_2SO_4 were used in this experiment since the results of experiment 2 indicated that greatest response was obtained from 0.1 percent of Na_2SO_4. The results of this experiment in general confirmed those of the previous experiments. Greater weight gains were obtained from the addition of 0.1 percent Na_2SO_4 (Table 4) than from the 0.05 percent level except when the diet contained 0.1 percent additional methionine, in which case no appreciable response was obtained from either level of sulfate. Analysis of the pooled data showed that the weight gains

TABLE 4.—*Mean body weight gains and feed conversion of broiler chicks fed combinations of methionine and/or sodium sulfate in a corn-soy diet to eight weeks of age (experiment 3)*

Methionine, %	Sodium sulfate in diet, % 0	0.05	0.10	Mean
	Body weight gains (g.)			
0	1438	1494	1528	1487[a]
0.05	1518	1531	1575	1541[b]
0.10	1581	1595	1592	1589[c]
Mean	1512[a]	1540[ab]	1565[bc]	
	Feed/gain			
0	2.40	2.28	2.29	2.32[a]
0.05	2.30	2.30	2.24	2.28[a]
0.10	2.20	2.17	2.16	2.18[b]
Mean	2.30[a]	2.25[b]	2.23[b]	

[1] Mean values in the same column or row with different superscripts are significantly different by Duncan's multiple range test, ($P < 0.05$).

were significantly greater than the control chicks receiving no supplemental sulfate. The weight gains of the chicks fed 0.05 percent Na_2SO_4 were intermediate and not significantly different from either those of the chicks fed 0.1 percent Na_2SO_4 or those fed no additional sulfate.

Feed conversion was generally improved in the treatment groups fed Na_2SO_4 even when the diets also included supplemental methionine (Table 4). Improved feed conversion, however, was not observed in experiment 2. Since different strains of chicks were used in the two experiments it is possible that the results obtained could be due to strain differences. There was a consistent improvement in feed conversion in all experiments when the basal groups were compared to the basal plus Na_2SO_4 groups. The feed conversion for the control groups was 2.25, 2.40, and 2.40 in experiments 1, 2, and 3, respectively, while the feed conversion of the groups fed the control plus Na_2SO_4 averaged 2.18, 2.34, and 2.29, respectively.

Experiment 4. Although the differences in

TABLE 5.—*Mean body weight gains and feed conversion of broiler chicks fed combinations of methionine and different sulfur compounds in a corn-soy diet for 17 days (experiment 4)*

Supplement	Body weight gains, (g.) Methionine added (%) 0	0.05	0.10	Feed/gain Methionine added (%) 0	0.05	0.10
None	264	272	278	1.70	1.63	1.61
0.05[1] Na_2SO_4	257	275	288	1.73	1.64	1.62
0.10[2] Na_2SO_4	263	287	283	1.71	1.60	1.60
0.05[1] $CaSO_4$	269	273	283	1.70	1.62	1.58
0.10[2] $CaSO_4$	256	265	282	1.69	1.62	1.64
0.05[1] $Na_2S_2O_3$	270	286	290	1.67	1.59	1.60
0.10[2] $Na_2S_2O_3$	269	281	284	1.63	1.60	1.59
0.05[1] $NaHSO_3$	264	279	286	1.71	1.62	1.59
0.10[2] $NaHSO_3$	275	273	285	1.69	1.61	1.61
0.05[1] $MgSO_4$	273	272	288	1.72	1.64	1.57
0.10[2] $MgSO_4$	278	281	285	1.67	1.59	1.58

[1] Equivalent to S in 0.05% Na_2SO_4.
[2] Equivalent to S in 0.10% Na_2SO_4.

body weight gain and feed conversion were not great enough to be statistically significant, the data summarized in Table 5 show that there was a fairly consistent response from most of the sulfur compounds. The least response seems to have been from $CaSO_4$ while most of the other compounds appeared at least equal to or better than Na_2SO_4 in stimulating body weight gains. The small response may be attributed to the shorter feeding period in this experiment. Since the sulfur compounds were fed at levels calculated to provide the same amount of sulfur as in the Na_2SO_4 treatments it would appear that some of the variability between compounds might be due to different absorption or solubility rates, or possibly to different modes of action once absorbed from the intestinal tract. The possibility that the response to these compounds might be due to the cations involved cannot be completely ruled out, although the basal diet was considered to meet the requirements for sodium, calcium, and magnesium. In addition, Ross and Harms (1970) obtained highly significant responses to Na_2SO_4 when the diet contained 0.88 percent of NaCl in addition to other sources of sodium.

The consistent response to the supplementation of Na_2SO_4 to a diet low in added sulfate and deficient in methionine in these four experiments confirms the results previously observed with chicks fed Na_2SO_4 in purified diets (Machlin, 1955; Gordon and Sizer, 1955; and Ross and Harms, 1970), and lends further support to the hypothesis that there is either a dietary requirement for sulfate *per se* or that sulfate spares methionine through conversion to cystine (Machlin *et al.*, 1954). The commonly accepted assumption that the total sulfur requirements of the chick can be met by the sulfur amino acids may be unjustified in the light of these results. If the sulfur needs of the chicks can be met through the oxidation of the sulfur amino acids it would appear to be an inefficient and wasteful process.

In view of the fact that inorganic sulfate is involved in the metabolism of many tissues and systems as well as in important detoxication mechanisms it seems reasonable to provide an adequate quantity of inorganic sulfur in the diet. On the basis of the results from this study it would appear advisable to use the sulfate forms of trace minerals commonly added to broiler diets or to add 0.1 percent sodium sulfate to provide an adequate level of sulfate.

SUMMARY

Four experiments were conducted with a total of 3336 broiler-type chicks fed a high energy corn-soy basal diet, low in added sulfate and deficient in methionine. In three experiments graded levels of DL methionine or sodium sulfate were fed for seven or eight weeks. Increased weight gains and improved feed efficiency were obtained from methionine supplementation confirming that the basal diet was deficient in this amino acid. Consistent increases in weight gains were also obtained from the addition of 0.05 to 0.5 percent of Na_2SO_4 to the basal diet, confirming previously reported results with purified diets. No response was obtained by increasing the level of supplementation of Na_2SO_4 above 0.1 percent.

In the fourth experiment, 17-day old chicks showed small but consistent increases in body weight when Na_2SO_4, $Na_2S_2O_3 \cdot 5H_2O$, $NaHSO_3$, and $MgSO_4$ were fed at rates to equal the sulfur content of 0.05 and 0.1 percent of Na_2SO_4. Equivalent quantities of $CaSO_4$ appeared to be without effect.

The hypothesis is advanced that there is either a dietary requirement for sulfate *per se* or that sulfate spares methionine through conversion to cystine.

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mococcus and then every 6 hours thereafter for 4 additional doses. The compound was without appreciable effect upon the local lesions, associated septicemia, etc., and both succumbed in 4 days.

Two rabbits were given *optochin base* suspended in oil by intramuscular injection in dose of 0.01 gm. per kilo immediately after intradermal inoculation with pneumococcus and repeated every 24 hours for 3 additional doses. Both succumbed about 4 days later with no appreciable effects upon the local lesions, associated septicemias, etc.

Two rabbits were given *ethyhydrocuprein hydrochloride* dissolved in water by intramuscular injection in dose of 0.01 gm. per kilo immediately after intradermal inoculation with pneumococcus and repeated daily for 2 additional doses. Both animals succumbed between 3 and 4 days after inoculation with no appreciable effects upon the local lesions, septicemias, etc.

Two rabbits were given *quinine* and *urea hydrochloride* by stomach tube in dose of 0.01 gm. per kilo immediately after intradermal inoculation with pneumococcus and the dose repeated every 6 hours for 6 additional doses. Two additional animals were given the same compound in the same dosage by intramuscular injection immediately after intradermal inoculation and repeated every 6 hours for 4 additional doses. All 4 animals succumbed in about 4 days with no appreciable effects upon the local lesions and associated septicemias, fever, leukocytosis, etc.

Two untreated controls inoculated intradermally at the same time developed the typical local lesions with daily positive cultures of edema fluids, daily positive blood cultures, leukocytic and febrile changes and succumbed between 4 and 5 days after inoculation.

Summary. Optochin base, ethyhydrocuprein hydrochloride and quinine and urea hydrochloride administered by stomach tube and by intramuscular injection in repeated doses had no appreciable curative effects upon the local lesions, associated septicemia, fever or leukocytic changes induced in rabbits by the intradermal inoculation (Goodner) of virulent type I pneumococcus.

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7571 P

Sulphates of Sodium and Magnesium on Gastro-intestinal Activity.

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Our experiments were designed to ascertain the effects of the sulphates of sodium and magnesium on the gastro-intestinal tract of unanesthetized dogs, each having a gastric and intestinal fistula. Rozen and Perussé[1] using unanesthetized dogs with a gastric fistula reported on gastric activity several hours after the use of magnesium chloride but other similar work has not been noted.

Two female dogs, S and B, were used as subjects. The fistulae were made after the manner of Thiry[2] (intestinal) and Carlson[3] (gastric) in a 2 stage operation. The animals were in good physical condition. Food was withheld for 18 hours prior to an experiment, but water was allowed *ad libitum*. Both subjects were trained to lie quietly during the experiments. Our experiments will be described under 2 series since the technic in each differed.

Series I—This series deals with the effects of the sulphates of sodium and magnesium on gastro-intestinal activity when the salines were placed in the stomach, graphic records (smoked paper) being obtained from both stomach and intestinal segment by means of balloons and soap water manometers. The salines were usually given after a normal record of about 1 hour had been procured.

The controls for this series were of 2 kinds: (a) with the stomach empty and (b) with the stomach containing 0.85% sodium chloride.

Results: Isotonic sodium or magnesium sulphate (25 to 50 cc. amounts) in contact with the gastric mucosa was without noteworthy effect on gastro-intestinal activity. Hypertonic (5 times) solutions of magnesium sulphate, however, depressed both gastric and intestinal movements slightly but were without effect on intestinal activity. Hypertonic sodium sulphate (5 times) appeared to increase intestinal activity about 30%.

Series II—Series II deals with the effects of the above salines on the gastro-intestinal activity of dogs S and B when the salines were

[1] Rozen, J. S., and Perussé, G. L., *Am. J. Physiol.*, 1929, **91**, 298.
[2] Thiry, *Sitzgsbr d. Wiener Acad. Math. Nat. Hist.*, 1864, **50**, 77.
[3] Carlson, A. J., "The Control of Hunger in Health and Disease," University of Chicago Press, 1916, 42.

placed in the loop of the intestinal fistula. As before, simultaneous records of the gastric and intestinal movements were taken, the gastric movements being recorded by the balloon method and the intestinal activity by a new closed system method in which the intestinal fistula was directly connected to the manometer. The essential parts in this system were: fistula—reservoir—manometer. A small stopper carrying a glass tube was placed in the opening of the fistula (dog B) or a rubber tube over a glass tube (dog S). Between the manometer and the fistula was a reservoir into which the solution from the intestinal fistula could flow when contractions occurred and from which the solution could drain into the segment of intestine when relaxation occurred. The manometer was the same type used in the first series. This method of recording intestinal activity we believe is essential, since there is no escape of fluid as in the balloon method, thus making long observations possible.

The results obtained were from experiments of 4 hours duration in which the sulphate solutions were either interspersed between physiologic saline or following it, each saline being left in the intestinal fistula for a period of about 1 hour. The hypertonic solutions were never more concentrated than twice hypertonic. Distilled water was also used in a few experiments.

Results: *Stomach*—no noteworthy effects.

Intestines—Hypertonic sodium chloride and distilled water: Distilled water in general caused only a slight increase in movements, except in 2 experiments in "B" the amplitude was doubled when used following isotonic sodium chloride. Hypertonic sodium chloride increased the amplitude over the normal but slightly in some instances, whereas in others the amplitude was increased 8 times that of the control, the average being about 3 times. There was also a temporary increase for a few minutes in the fluid content of the intestines.

Isotonic sodium sulphate and isotonic magnesium sulphate: The isotonic sodium sulphate stimulated the activity to twice that of the control sodium chloride regardless of whether it preceded or followed the magnesium sulphate. The magnesium sulphate stimulated the activity to 4 times that of the control. When isotonic sodium chloride followed isotonic magnesium sulphate the usual basic effects of isotonic sodium chloride were absent. Both isotonic magnesium and sodium sulphate increased the fluid content of the

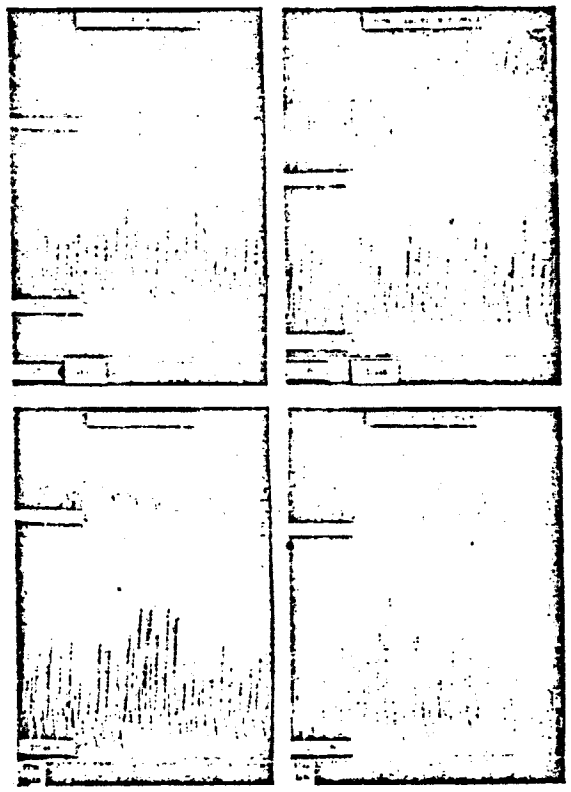

FIG. 1.

Effects of various isotonic salines on gastric intestinal loop activity on unanesthetized dog (Brindle: 12½ kg. Female. 12/22/33.)

Time in 6 seconds: Tracings as follows: Upper left, isotonic sodium chloride; upper right, isotonic magnesium sulphate; lower left, isotonic sodium chloride; lower right, isotonic sodium sulphate.

The upper tracings were taken one-half hour, lower tracings about 45 minutes, after the salines were placed in the intestinal loop.

intestines, the effects lasting throughout the experiment. The magnesium effect was the more marked.

Hypertonic sodium sulphate and hypertonic magnesium sulphate: The magnesium sulphate (twice hypertonic) caused a stimulation of intestinal activity 9 to 10 times that of the control (isotonic sodium chloride). The sodium sulphate (twice hypertonic) increased the activity about 8 times when it preceded the magnesium, and about 12 times when it followed the magnesium.

When isotonic sodium chloride followed hypertonic magnesium sulphate the usual contractions present during sodium chloride were absent in some experiments and present in others. The fluid content of the intestine was markedly increased with each of the hypertonic solutions but slightly more so after the magnesium. The magnesium also appeared to lower the tone of the intestine.

Specific effects of both magnesium and sulphate ions have thus been demonstrated as well as the usual osmotic effects.

7572 P

Determination of Lactic Acid in Presence of Certain Interfering Substances.

EVAN W. MC CHESNEY. (Introduced by W. deB. MacNider.)

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Probably the best technique now available for the determination of lactic acid is that of Friedemann, Cotonio, and Shaffer[1] as modified by Friedemann and Kendall[2] and, more recently, by Friedemann and Graeser.[3] There are certain substances which interfere with the lactic acid determination; these have been listed in the original and some of the subsequent articles. Some of these substances, notably the sugars, may be removed from solution by the copper sulfate-calcium hydroxide procedure.[2] Appreciable amounts of others, however, remain in solution even after this treatment. Important examples of these substances are malic and citric acids, both of which give large yields of bisulfite-binding substances under the conditions used for the oxidation of lactic acid to acetaldehyde. In fact, by some slight modifications of the conditions it is possible to obtain very nearly a quantitative yield of acetaldehyde from malic acid (90 to 93%).[4] The product formed by the oxidation of citric acid is apparently acetone[5] and by the lactic acid method a yield of about 60% of the theoretical may be obtained.[4] However, both malic and citric acids may be quite completely removed from solution by means of basic lead acetate as follows: An amount of solution thought to contain 20-50 mg. of lactic acid and any reasonable amount of the interfering substance is placed in a 100 cc. volumetric flask, and diluted to a volume of about 50 cc. A drop of aqueous phenol red is added, then 10% NaOH is added drop by drop until the solution is neutral followed by one drop excess. A saturated solution of basic lead acetate (Haden) is added in 5 cc. portions until there is an excess; usually 5 cc. is sufficient and a large excess is to be avoided. Zinc ions should be absent from the solution as some precipitation of lactic acid seems to result if they are present. The solution is now diluted to the mark, mixed, and filtered. Lactic acid is determined in the usual way on aliquot portions of the filtrate. With the addition of the $MnSO_4$-H_3PO_4 reagent, a precipitate forms and this causes some bumping during the subsequent boiling but does not seem to interfere otherwise. Table 1 shows the completeness of the separation of lactic and malic acids by this method.

[1] Friedemann, T. E., Cotonio, M., and Shaffer, P. A., *J. Biol. Chem.*, 1927, **73**, 335.

[2] Friedemann, T. E., and Kendall, A. I., *J. Biol. Chem.*, 1929, **82**, 23.

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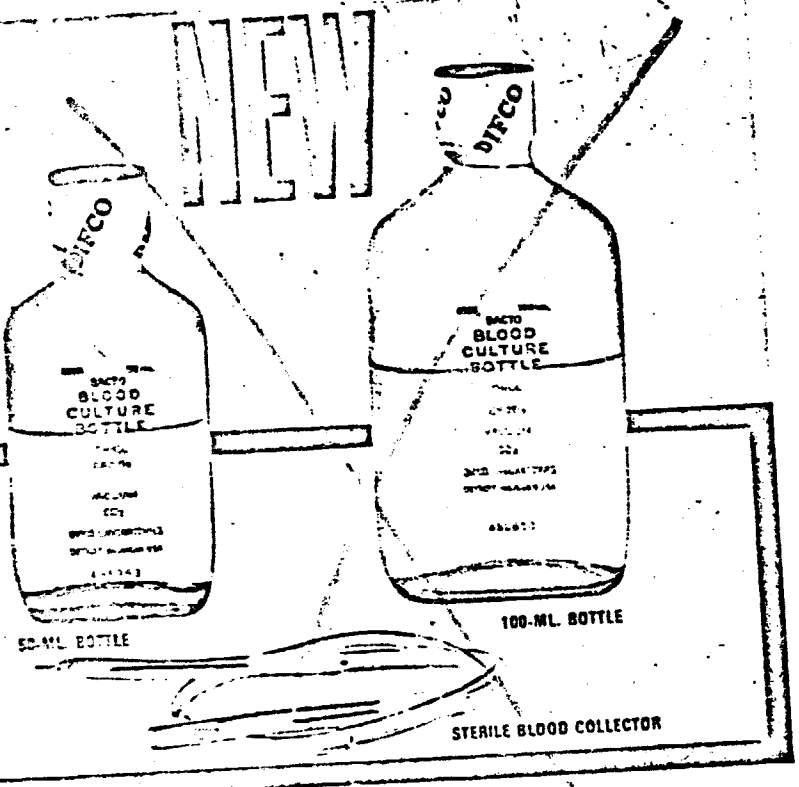
[5] Kuyper, A. C., *J. Am. Chem. Soc.*, 1933, **55**, 1722.

TABLE I.

Mg. lactic acid in 100 cc. vol.	Mg. malic acid added	Mg. lactic acid found
40.8	0	40.8
0	100	0.8
20.4	100	20.3
40.8	100	40.1
81.6	100	78.6
122.4	100	119.4
40.8	20	40.2
40.8	50	40.0
40.8	75	40.2

The average recovery of lactic acid from the mixtures was 98%.

Other substances. Of the other substances reported in the original article [1] to interfere with the lactic acid determination, the following have been found to give precipitates with basic lead acetate when 10 mg. of the substances are present in the final volume of 100 cc.: tartaric acid, citric acid, tyrosine, cystine, and maleic acid. The following have been found to yield precipitates with basic lead acetate when 5 mg. of the substance are present in the final volume of 100 cc.: tartaric acid, citric acid, tyrosine, and cystine (trace). The sugars glucose, fructose, xylose, galactose, and arabinose are of course not precipitated. This method is not likely to be of value in eliminating the various interfering alpha-hydroxy acids since in addition to lactic acid, alpha-hydroxy isovalerianic acid is not precipitated, nor is the acid derived from the deamination of leucine (alpha-hydroxy isocaproic acid).



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The effect of Na_2SO_4 on urinary acidification in chronic renal disease

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The effects of Na_2SO_4 on the pattern of urinary acid excretion were studied in 6 normal subjects and 14 patients with chronic renal disease when the serum bicarbonate concentration was normal and reduced. The infusion of Na_2SO_4 into patients with renal disease resulted in a rise in urine pH and bicarbonate excretion when the serum bicarbonate was normal. In contrast, normal subjects consistently excreted a more acid urine in response to Na_2SO_4. During metabolic acidosis, the infusion of Na_2SO_4 in the patients resulted in urinary acidification. These findings suggest that in renal failure nephron overperfusion will flood the distal tubule with $NaHCO_3$ and impair urinary acidification even when a potent acidifying salt such as Na_2SO_4 is given. The inability of Na_2SO_4 to acidify the urine of the diseased kidney indicates that this is not an expression of intrinsic tubular damage and this is seen in the effects of metabolic acidosis when Na_2SO_4 is able to elicit maximum urinary acidification. The findings permit a tentative reconstruction of some of the factors responsible for the metabolic acidosis of renal failure.

Uremic acidosis must be the consequence of the failure of the kidney to excrete metabolically produced hydrogen ions. This may be manifested as the decreased excretion of ammonia or titratable acid or decreased reabsorption of bicarbonate. Early studies by Palmer and Henderson[1] demonstrated that patients with chronic renal acidosis could excrete very acid urines. Nevertheless,

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the excretion of ammonia was strikingly diminished, a finding repeatedly confirmed by other investigators.[2-8] The low ammonia excretion in the presence of a low urine pH had been attributed principally to a reduction in nephron mass.[5-7]

Evidence is controversial concerning the capacity to form titratable acid and reabsorb bicarbonate. Wrong and Davies[5] found that titratable acid excretion was lower in patients with renal disease than in normal subjects. However, this may have been the result of a lower protein intake in experimental subjects who were ill as a result of which buffer excretion was reduced. It is known that titratable acid excretion is a function principally of urine pH and the excretion of the major buffer anion, phosphate. Briggs and associates[8] demonstrated that, when the same quantity of dietary protein was ingested by patients with renal disease and normal subjects, titratable acid excretion was the same.

Acute infusions of bicarbonate given to patients with uremic acidosis disclose a normal bicarbonate reabsorptive capacity without evidence of excessive spray.[9] On the other hand, in balance studies, a small bicarbonate leak can frequently be demonstrated when the serum bicarbonate is near normal.[10] Schwartz and coworkers[10] emphasized that the complete reabsorption of bicarbonate and the formation of a highly acid urine containing maximal titratable acid is achieved in renal disease only at the expense of a considerable fall in serum bicarbonate. They interpreted this finding as indicative of a tubular defect in hydrogen secretion. An alternate explanation would attribute impaired bicarbonate reabsorption to over perfusion of intact nephrons of the diseased kidney.

In the present studies, the ability of patients with chronic renal disease to acidify the urine maximally in response to an infusion of hypertonic sodium sulfate was examined. It is known that sodium sulfate causes intense acidification in a normal subject given sodium-retaining steroids,[11] probably because the reabsorption of sodium in the presence of an impermeant anion raises the distal transtubular potential difference, thereby trapping the secreted hydrogen.[12] Under these conditions the normal kidney may excrete urine with a pH as low as 4.0.

The data on our experimental subjects with renal disease disclose that when the serum bicarbonate is normal, the infusion of sodium sulfate raises urine pH and lowers titratable acid excretion. This is in contrast to the uniform acidification of the urine produced by Na_2SO_4 infusion in normal subjects. On the other hand, when the serum bicarbonate in patients with renal disease is reduced, urine pH falls and titratable acid excretion rises. The alkalinization of the urine by Na_2SO_4 in patients with renal disease when the serum bicarbonate is normal is attributed to nephron over perfusion and osmotic diuresis rather than tubular damage. The fact that the diseased kidney can acidify the urine maximally when the serum bicarbonate is reduced indicates that tubular function is well preserved.

Methods

Studies were performed on 14 patients with chronic renal disease. Glomerular filtration rates, determined by endogenous creatinine clearance, ranged from 4 to 25 ml. per minute. The diagnoses in this group of patients included arteriolar nephrosclerosis (9 patients), chronic glomerulonephritis (4 patients), and lupus nephritis (1 patient). All patients had previously developed metabolic acidosis while on an ad libitum diet.

The effect of an infusion of sodium sulfate on urinary acidification was studied during metabolic acidosis (10 patients) and when the serum bicarbonate was normal (10 patients). Six of the 10 patients were studied both before and after the correction of metabolic acidosis. Correction of acidosis was accomplished with Shohl's solution until serum bicarbonate was greater than 26 mEq. per liter. Shohl's solution was then discontinued for 48 hours before the sodium sulfate infusion. Studies in acidosis were performed during either spontaneous or induced (ammonium chloride administration) metabolic acidosis. When acidosis was induced by ammonium chloride, the latter was discontinued 48 hours before the infusion of sodium sulfate.

All patients were given exogenous salt-retaining hormone before the study, either 9-α-fluorohydrocortisone, 1 mg. orally in divided doses over a 12 hour period, or desoxycorticosterone, 5 mg. intramuscularly 12 hours and again 2 hours before the study.

Studies were performed in the morning during fasting. Venous blood was drawn at the beginning and end of the study for determination of sodium, potassium, CO_2 content, and creatinine. Patients were given 1 L. of water to drink over 40 minutes to insure adequate hydration. Urine was then collected at 30 minute intervals for the next 3 hours. The first two collection periods served as controls. At the end of the second control period, an infusion of 1 L. of 4 per cent sodium sulfate was started and infused rapidly during the following 2 hours. Sodium bicarbonate was added to the infusion in a concentration of approximately 5 mEq. per liter below the serum bicarbonate concentration to prevent dilution acidosis secondary to the rapid infusion of sodium sulfate. Patients were recumbent during the study, standing, if necessary, only to void. An indwelling Foley catheter was used in 8 studies.

Urine collections were made either by voiding into a graduated cylinder with a thin layer of mineral oil or by aspirating into an oiled syringe if a catheter was in place. After measurement of the urine volume, an aliquot was transferred to a test tube containing several milligrams of phenyl mercuric nitrate to inhibit bacterial growth and layered with mineral

Table I. Effect of sodium sulfate infusions on acid excretion in normal subjects

Patient	Age	Sex	Period	GFR C_{cr} (ml./minute)	Plasma CO_2 content (mEq./L.)	Urine pH	Urine T.A.* (μEq/minute)	Urine NH_4^+ (μEq/minute)	Urine HCO_3^- (μEq/minute)	Urine Net acid† (μEq/minute)
H. F.	62	M	Control	110	28.8	6.35	17	23	6	34
			Na_2SO_4		28.1	4.25	45	51	0	96
P. M.	26	M	Control	90	26.0	5.68	24	27	0	51
			Na_2SO_4		25.1	4.15	45	34	0	79
C. N.	42	M	Control	120	26.8	6.25	10	33	3	40
			Na_2SO_4			4.45	27	61	0	88
J. O.	24	M	Control	140	28	6.42	31	28	50	9
			Na_2SO_4			4.66	36	47	0	83
S. I.	45	F	Control	90	24.1	4.90	18	15	0	33
			Na_2SO_4		23.9	4.15	38	26	0	64
A. M.	40	M	Control	100	28	6.70				
			Na_2SO_4			4.30				
Mean				108	27.0	6.05	20.0	25.2	12	33
					25.7	4.32	38.2	43.8	0	82

*T.A. = Titratable acid.
†Net acid = T.A. + NH_4^+ − HCO_3^-.

oil. These aliquots were then placed in a refrigerator at 4° C. Laboratory determinations, performed within 1 hour of the completion of the study, included pH, titratable acid, ammonium, and urine bicarbonate. In 1 patient studied during metabolic acidosis, only urine pH was determined.

For purposes of comparison, the response to sodium sulfate infusion of 6 normal subjects was studied. In one of these subjects, only urine pH was determined. All had a creatinine clearance in excess of 90 ml. per minute, and all were in a state of normal acid-base balance.

In addition, the response of renal acid excretion to infusion of 5 per cent mannitol was examined in 7 of the patients with renal disease during metabolic acidosis, and in 3 of the patients after correction of acidosis. These studies were performed in the same fashion as the studies with sodium sulfate.

Table II. Effect of sodium sulfate infusions on acid excretion in patients with renal disease when plasma bicarbonate was normal and low

					Plasma	
Patient	Age	Sex	Diagnosis	Period	GFE (C_{cr}) (ml./minute)	Plasma CO_2 content (mEq./L.)
J.T.	58	M	Arteriolar nephrosclerosis	Control	24	28.9
				Na_2SO_4		27.9
Z.H.	56	F	Arteriolar nephrosclerosis	Control	9	25.8
				Na_2SO_4		25.6
M.C.	32	M	Chronic glomerulonephritis	Control	20	26.7
				Na_2SO_4		24.8
W.J.	19	M	Accelerated hypertension	Control	18	27.0
				Na_2SO_4		27.9
F.S.	22	F	Lupus nephritis	Control	13	25.1
				Na_2SO_4		
J.R.	62	M	Arteriolar nephrosclerosis	Control	20	26.3
				Na_2SO_4		26.5
M.T.	25	M	Chronic glomerulonephritis	Control	8	27
				Na_2SO_4		
C.E.	60	M	Arteriolar nephrosclerosis	Control	4	27.0
				Na_2SO_4		25.6
S.F.	63	F	Arteriolar nephrosclerosis	Control	12	26.1
				Na_2SO_4		25.4
C.L.	52	F	Arteriolar nephrosclerosis	Control	5	28.4
				Na_2SO_4		27.6
M.B.	55	F	Arteriolar nephrosclerosis	Control	6	
				Na_2SO_4		
R.F.	60	F	Chronic glomerulonephritis	Control	6	
				Na_2SO_4		
I.S.	64	F	Arteriolar nephrosclerosis	Control	24	
				Na_2SO_4		
C.R.	75	M	Arteriolar nephrosclerosis	Control	18	
				Na_2SO_4		
Mean				Control	13.4	26.8
				Na_2SO_4		26.4

*T.A. = Titratable acid.
†Net acid = T.A. + NH_4^+ − HCO_3^-.

Glomerular filtration rate was estimated from endogenous creatinine clearance; creatinine in urine and blood was determined on the Technicon Auto Analyzer. Urine pH was measured with a Beckman pH meter. Titratable acid was measured by titration to a pH of 7.2 during metabolic acidosis and 7.4 when the serum bicarbonate was normal. Urinary ammonia and CO_2 content of serum and urine were measured by methods previously described.[13]

Results

Normal subjects. Table I summarizes the response of 6 normal subjects to an infusion of sodium sulfate. Following the infusion of sodium sulfate, urine pH fell sharply in every instance to levels below 4.7. The excretion of both am-

Plasma bicarbonate, normal						Plasma bicarbonate, low					
	Urine						Urine				
pH	T.A.* (μEq/minute)	NH_4^+ (μEq/minute)	HCO_3^- (μEq/minute)	Net Acid† (μEq/minute)	Plasma CO_2 content (mEq./L.)	pH	T.A.* (μEq/minute)	NH_4^+ (μEq/minute)	HCO_3^- (μEq/minute)	Net Acid† (μEq/minute)
6.10	9	6	2	13	18.9	5.22	18	26	10	34
6.65	8	8	12	4	19.3	4.50	29	30	0	59
6.46	9	6	9	6	13.2	5.01	13	6	0	19
6.84	7	5	17	−5	13.3	4.60	17	7	0	24
5.76	14	7	1	20	20.0	5.02	21	8	2	27
7.02	7	10	42	−25	–	4.82	29	12	1	40
6.79	7	1	11	−3	15.4	5.10	29	10	1	38
7.08	4	1	23	−18	14.8	4.90	36	12	0	48
6.51	1	3	6	−2	10.3	4.42	18	6	0	24
6.75	1	4	22	−17	10.0	4.41	23	7	0	30
5.80	3	2	1	4	14.6	4.92	6	3	0	9
6.40	5	3	7	1	15.2	4.60	20	8	0	28
6.65	7	10	8	9						
6.83	11	10	26	−5						
5.75										
7.09										
5.40	12	5	1	16						
6.52	8	4	11	1						
6.43	3	2	3	2						
6.82	3	2	17	−12						
					15.0	5.82	9	3	4	8
					15.1	6.20	9	3	6	6
					12.0	5.34	9	4	0	13
					12.0	4.82	12	5	0	17
					15.5	5.04	17	19	1	35
					15.7	4.40	26	22	0	48
					18.0	5.89	12	9	2	19
					18.1	5.32	15	11	1	25
6.17	7	4.7	4.7	7	15.3	5.17	15.2	9.4	2	23
6.80	6	5.2	19.7	−8.4	14.8	4.83	21.6	11.7	1	32.5

monia and titratable acid usually doubled. This pattern of acid excretion was achieved rapidly, usually by the end of the first hour of sodium sulfate infusion, and persisted throughout the study.

Patients with renal disease, sodium sulfate infusion.

SERUM BICARBONATE, NORMAL. Table II summarizes the response of 10 patients with advanced renal disease to an infusion of sodium sulfate when the serum bicarbonate was normal. In striking contrast to the normal subjects, urine pH rose in all instances. This was associated with a sharp rise in bicarbonate excretion. The excretion of ammonia and titratable acid was low during the control period and did not change consistently following sodium sulfate infusion. In consequence, net acid excretion consistently fell.

SERUM BICARBONATE BELOW 20 MEQ. PER LITER. Table II also lists the response of 10 patients with advanced renal disease to an infusion of sodium sulfate when

Table III. Effect of mannitol infusions on acid excretion in patients with renal

		Plasma bicarbonate, normal				
				Urine		
Patient	Period	GFR (C_{cr}) (ml./minute)	Plasma CO_2 content (mEq./L.)	pH	T.A.* (μEq/minute)	NH_4^+ (μEq/minute)
J. T.	Control	24	28.7	5.35	18	14
	Mannitol		29.3	6.35	11	13
M. C.	Control	20	25.8	5.87	27	18
	Mannitol		25.0	5.97	23	20
W. J.	Control	18	25.4	6.40	13	8
	Mannitol		24.0	6.73	12	6
Z. H.	Control	9				
	Mannitol					
R. F.	Control	6				
	Mannitol					
C. R.	Control	18				
	Mannitol					
L. S.	Control	24				
	Mannitol					
M. B.	Control	6				
	Mannitol					
J. R.	Control	8				
	Mannitol					
Mean	Control	14.8	26.6	5.87	19.3	13.3
	Mannitol		26.1	6.35	15.3	13.0

*T.A. = Titratable acid.
†Net acid = T.A. + NH_4^+ - HCO_3^-.

the serum bicarbonate was low. In every instance the control urine pH was lower during acidosis than when the serum bicarbonate was normal. The lowest urine pH was 4.42 in a patient whose serum bicarbonate was 10 mEq. per liter. In only 2 of 10 patients was the control urine pH above 5.3. The ability to elaborate a very acid urine when serum bicarbonate is reduced confirms previous observations.[5,10]

Despite the low control urine pH, 9 of 10 patients responded to sodium sulfate with further acidification. Titratable acid excretion rose significantly in 9 of 10 patients and remained unchanged in 1 patient following sodium sulfate infusion. Ammonia excretion usually rose slightly as urine pH fell.

Patients with renal disease, mannitol infusion.

SERUM BICARBONATE, NORMAL. Three patients were studied with mannitol infusion when the serum bicarbonate was normal (Table III). All 3 responded

disease when plasma bicarbonate is normal and low

		Plasma bicarbonate, low					
			Urine				
HCO_3^- (μEq/minute)	Net acid† (μEq/minute)	Plasma CO_2 content (mEq./L.)	pH	T.A.* (μEq/minute)	NH_4^+ (μEq/minute)	HCO_3^- (μEq/minute)	Net acid† (μEq/minute)
2	30	19.2	5.05	24	39	0	63
10	14	19.8	5.39	16	28	1	43
2	43						
4	39						
7	14						
23	-5						
		20.1	6.40	11	6	8	9
		20.2	6.40	9	6	7	8
		13.8	5.44	6	4	0	10
		13.7	5.58	11	5	2	14
		15.2	5.36	17	19	0	36
		16.2	5.36	19	20	0	39
		13.6	4.88	22	15	0	37
		15.2	5.00	20	19	0	39
		15.8	5.33	15	3	0	18
		15.7	5.90	12	3	1	14
		15.4	4.89	17	8	0	25
		15.0	4.95	12	6	0	18
3.7	29.0	16.2	5.34	16.0	13.4	1	28.3
12.3	16.0	16.5	5.51	14.2	12.4	1.6	25.0

with a rise in urine pH (mean pH change 5.87 to 6.35), and with a greater than threefold increase in urine bicarbonate excretion. Net acid excretion fell sharply in all 3 patients.

SERUM BICARBONATE, LOW. Seven patients were studied with mannitol infusion when the serum bicarbonate was below 20 mEq. per liter (Table III). In 3 of the 7 essentially no change in urine pH occurred. In the remaining 4, a small but significant rise in urine pH occurred (mean pH change 5.13 to 5.45). Data for these mannitol studies are given in Table III.

Discussion

The surviving nephrons in renal disease are exposed to tremendous osmotic diuresis. This follows from the fact that in the steady state and on a normal diet patients with renal disease must excrete the same load through a greatly diminished number of nephrons as do normal subjects.[14] In our patients the GFR approximated a mean value of about 15 ml. per minute. If the normal GFR is 130 ml. per minute the solute load per nephron in the diseased kidney must be increased at least ninefold. However, since elevation of the GFR per nephron is the usual accompaniment of reduced nephron mass,[15] it is likely that a GFR of 15 ml. per minute over estimates the number of surviving nephrons by about 60 per cent. As a rough approximation, then, it is probable that each surviving nephron in our patients was subjected to a solute load some 15 to 20 times greater than normal.

The mechanism for the increased solute excretion per nephron involves three factors: increased GFR per nephron; increased plasma concentration of nonreabsorbable solutes (SO_4, urea, etc.); and suppression of fractional reabsorption of filtrate in the proximal tubule as a result of increased quantities of nonreabsorbable solute. In consequence, large quantities of filtrate issue out of the proximal nephron, flooding the more distal segments with a relatively alkaline fluid. Although osmotic diuresis alone does not raise the concentration of HCO_3^- at the end of the proximal tubule above that of the nondiuretic animal,[16] we have recently shown that suppression of proximal reabsorption by expansion of effective extracellular volume increases the concentration of HCO_3^- in the fluid leaving the proximal tubule.[17] If some degree of overexpansion of extracellular volume is a feature of advancing renal failure, then it is likely that the increased quantities of tubular fluid which flood the distal nephron will contain HCO_3^- at an elevated concentration. In consequence, the intrinsic ability of this segment to perform its acidifying functions may be completely masked; the latent capacity for net hydrogen excretion (titratable acid plus ammonia minus bicarbonate) and for the generation of pH gradients can not emerge when distal hydrogen secretion, though perhaps greatly increased, is expended on bicarbonate reabsorption.

This is illustrated in the paradoxical alkalinization of the urine that follows the administration of sodium sulfate to patients with renal disease when the serum bicarbonate is normal. Sodium sulfate is perhaps the most potent stimulus to distal acidification. In normal subjects (Table I) urine pH can be depressed as low as 4.15 and net acid excretion enormously augmented in the face of a normal serum bicarbonate when sodium sulfate is given. By contrast urine pH was elevated and net acid excretion depressed when sodium sulfate was infused in patients with renal disease whose serum bicarbonate was normal. It seems probable that in these patients, the already prevailing osmotic diuresis sweeps sufficient bicarbonate out of the proximal tubule virtually to saturate the distal hydrogen secretory capacity. The additional delivery of bicarbonate to the distal nephron as a result of the sodium sulfate load overwhelms the hydrogen secretory system, so that the acidifying effect of sodium sulfate, clearly evident in the normal subjects, was completely masked, and the urine became relatively alkaline. This interpretation is borne out by the tendency of mannitol diuresis to alkalinize the urine in patients with renal disease. Infusion of 5 per cent mannitol caused a rise in urine pH in 3 patients studied when their serum bicarbonate was normal, and caused a rise in urine pH in 3 of 6 patients studied during metabolic acidosis. The results indicate that an increased osmotic load in a patient with renal disease tends to lower bicarbonate reabsorption.

These considerations indicate that the ability of the distal nephron to acidify the urine in renal disease cannot be examined when the serum bicarbonate is normal. Only during metabolic acidosis can the delivery of bicarbonate to the distal nephron in the presence of the massive osmotic diuresis characteristic of renal failure be reduced sufficiently to permit the latent acidifying capacity to express itself.

In patients with renal disease, urinary acidification was augmented when serum bicarbonate was reduced from normal to low values (Table II); urine pH fell sharply (in one instance to 4.42), titratable acid excretion increased greatly, and ammonia excretion rose to a modest extent. When distal acidification was maximally stimulated by an infusion of sodium sulfate during metabolic acidosis (Table II), urine pH usually fell to values below 5.0; titratable acid excretion increased but ammonia excretion either did not change or rose only slightly.

These findings suggest that the intrinsic ability of the distal nephron to acidify the urine in renal disease is remarkably well preserved. When metabolic acidosis develops, urine pH can be lowered to values quite comparable to those achieved by normal subjects. Further stimulation of hydrogen secretion by administration of sodium sulfate elicits a further fall in urine pH. Since the urine pH is maximally depressed, it is obvious that the magnitude of the rise in titratable acid is limited only by the excretion of buffer. Since the excretion of buffer in the steady state is a direct function of the dietary intake, it follows that in metabolic acidosis patients with renal disease can eliminate the same amount of titratable acid as can normal subjects on the same diet. Therefore once bicarbonate is eliminated from the urine by lowering the serum bicarbonate there is no detectable defect in the capacity of patients with renal disease to form titratable acid.

The excretion of ammonia rose only minimally following sodium sulfate infusion in patients with renal disease. In a number of patients (Table II, Z. H., F. S., J. R., M. B., and R. F.) ammonia excretion did not exceed 10 μEq per

minute under any circumstances. Since urine pH was maximally depressed and titratable acid excretion maximally stimulated, the very low values for ammonia excretion cannot be the consequence of a defect in the ability of the distal nephron to generate pH gradients or to secrete hydrogen, but rather an intrinsic limitation of the ammonia-producing enzyme systems. This limitation, however, does not necessarily mean that there is nephron damage. It is possible that each surviving nephron is responding normally and maximally; the low ammonia excretion may, as others have suggested[5-7] simply reflect a shrinkage of nephron mass.

An alternative explanation for the failure of Na_2SO_4 to acidify the urine in patients with renal disease when the serum bicarbonate is normal involves the assumption that there is tubular damage which reduces the capacity to secrete hydrogen. According to this view the distal delivery of bicarbonate is normal. The capacity to secrete hydrogen and to reabsorb bicarbonate is reduced and therefore near saturation; consequently, the increased delivery of bicarbonate induced by sodium sulfate infusions alkalinizes the urine despite the fact that Na_2SO_4 is a potent stimulus for hydrogen secretion. When the serum bicarbonate is reduced, thereby lowering distal bicarbonate delivery, the distal secretory system is working so far below saturation that urine pH is sharply diminished; sodium sulfate can acidify the urine still further, even though the total capacity to secrete hydrogen is abnormally low.

It is not possible from these data definitively to exclude the hypothesis of tubular damage. Nevertheless, several lines of argument make it less attractive as an explanation for defective acidification than the foregoing thesis of overperfusion. It has been clearly demonstrated by bicarbonate titration studies that the total capacity to reabsorb bicarbonate (expressed as milliequivalents per 100 ml. GFR) in patients with renal disease is normal.[8] Since GFR per nephron is increased,[15] this means that maximum bicarbonate reabsorption per nephron is also increased. Not only is the total capacity to secrete hydrogen per nephron increased in renal disease, but in addition the ability to lower urine pH maximally is completely preserved. This is best demonstrated by the low urine pH obtained when Na_2SO_4 is infused during metabolic acidosis. In view of the elevated bicarbonate reabsorptive capacity per nephron and the intact intrinsic ability to establish maximum pH gradients, it seems reasonable to suppose that the impaired ability to acidify the urine maximally when the serum bicarbonate is normal, is the consequence of excessive bicarbonate loads per nephron rather than tubular damage.

A tentative reconstruction of the renal factors leading to metabolic acidosis in chronic renal disease, on the basis of this hypothesis, may be ventured. The diminished nephron mass results in greatly reduced ammonia production, thereby rendering net acid excretion critically dependent on the ability to form titratable acid. Titratable acid excretion is a function of the pH and buffer content of the urine. Since the buffer load per nephron is enormously increased, titratable acid excretion becomes almost entirely dependent on urine pH. When serum HCO_3^- is normal, HCO_3^- delivery to the distal tubule is so great that urine pH is relatively high (Table II); consequently, titratable acid excretion

is reduced and metabolic acidosis results. The serum HCO_3^- will stabilize (at any given rate of metabolic acid production in excess of that titrated by body buffers[18-20]) when the concentration falls sufficiently to allow more complete proximal reabsorption. This permits the distal nephron to generate very low urine pH and thereby facilitate the formation of maximal amounts of titratable acid.

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THE INFLUENCE OF NON-REABSORBABLE ANIONS ON ACID EXCRETION

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SUMMARY

1. Balance studies have been carried out to evaluate the influence of non-reabsorbable anion on the urinary excretion of acid and the plasma bicarbonate level.

2. Normal human volunteers were given a constant diet containing approximately 10–20 mEq of sodium and chloride and were infused each day with isotonic salt solutions containing an additional 155 mEq of sodium. During control periods all of the infused anion was chloride, while during experimental periods the chloride was replaced by equivalent amounts of sulphate. In this way it was possible to vary the nature of the anion load without changing the intake of sodium.

3. Substitution of sulphate for chloride caused negligible changes in urine acidity and net acid excretion and no significant change in plasma bicarbonate concentration.

4. It is concluded that under normal circumstances the nature of the anion load is not important in the regulation of acid excretion or the maintenance of the renal threshold for bicarbonate.

5. The suggestion is made that renal acid–base regulation is accomplished by the interaction of independent mechanisms that determine the bicarbonate threshold, the excretion of fixed anions and cations, and the synthesis of ammonia.

The physiological mechanisms regulating the renal bicarbonate threshold and the excretion of acid have not been fully elucidated. The possible importance of the anion load was suggested by the experiments of Schwartz, Jenson & Relman (1955), who reported that the rapid infusion of sodium sulphate into normal subjects produced immediate and intense acidification of the urine, provided that the subjects had been stimulated to reabsorb sodium by previous administration of adrenal steroids or ingestion of sodium-free diets.

Several studies since then have shown that acute loading with certain unreabsorbable anions increases the excretion of titratable acid (Bank & Schwartz, 1960), raises the renal threshold,

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but not the *Tm*, for bicarbonate (Rector & Seldin, 1962), and in the rat greatly increases the transtubular potential gradient (Clapp, Rector & Seldin, 1962). This work seemed to suggest that the replacement of chloride in the tubular lumen by a less reabsorbable anion, limits the number of negative charges that can move out of the tubule down the electrical gradient established by the active transport of sodium. As a result the lumen becomes more electronegative, and the inward secretion of hydrogen and potassium ions in exchange for sodium is thereby favoured.

During the past few years this concept has been extended to more chronic situations by the work of Schwartz and his colleagues (Gulyassy, van Ypersele de Strihou & Schwartz, 1962; Needle, Kaloyanides & Schwartz, 1964; Atkins & Schwartz, 1962; Kassirer *et al.*, 1965). They have demonstrated, in a variety of experimental conditions characterized by limitation or depletion of chloride, that the renal threshold for bicarbonate will be elevated as long as the supply of reabsorbable anion in the tubule is restricted. The renal bicarbonate threshold falls, and alkalosis is corrected, only when chloride—a reabsorbable anion—is restored to the diet. These workers suggest that the stimulus for the renal excretion of the endogenous acid load in steady states of chronic alkalosis may reside in the nature of the anions generated by metabolic processes, since all of these anions (sulphate, phosphate and various organic anions) are poorly reabsorbable and might stimulate the exchange of sodium for hydrogen on this basis alone (Tannen, Bleich & Schwartz, 1966).

A sustained influence of anions upon acid excretion and bicarbonate threshold has been demonstrated only under conditions of sodium or chloride depletion or in circumstances that enhance the tubular reabsorption of sodium. It remains to be determined whether the nature of the anion load influences renal acid–base regulation under the more usual circumstances in which the diet contains sodium and chloride. Does the kidney excrete acid in response to endogenous acid production simply because the anions of endogenous acids are less reabsorbable than chloride? The present studies were designed to answer this question.

METHODS

A total of eight balance studies were performed on six healthy volunteers who were permitted normal quiet activity while confined to a metabolic ward. A constant diet, low in sodium and chloride but of otherwise normal composition, was given throughout each balance study. Fluid was allowed *ad libitum*. The diet was supplemented with 155 mEq sodium chloride daily during a preliminary equilibration period of 4–6 days before starting balance collections.

In six studies, at the start of the balance period, oral salt supplements were withdrawn, and in their place an infusion of isotonic sodium chloride or sodium sulphate solution was given intravenously over 8 hr each day for the duration of the balance study. Thus, sodium intake and the dietary constituents responsible for acid production were held constant. Only the anion given with sodium was varied: chloride was infused for approximately 1 week, followed immediately by a week during which sulphate was administered. In four of the six balance studies there were additional periods of alternating chloride and sulphate infusion. In two of the six balances the diet was supplemented throughout the study with oral methionine, 6 g/day, in order to increase endogenous acid production.

In two balance studies the protocol was the same as that described above for the four balances

without methionine except that: (a) the infusions were administered continuously over 24 hr, and (b) the periods of administration of sulphate or chloride were only 3 days each. The general plan of the experimental protocol is illustrated in Fig. 1.

The isotonic saline used in each balance came from a single commercial lot (Abbott). The volume and sodium concentration of these infusions were measured and were found to be constant. An amount of isotonic sodium sulphate sufficient for one balance study was prepared as a single solution, and aliquots of 155 mEq were autoclaved. The sodium concentration of each infusion was measured. Subjects were allowed to sleep, sit in a chair, or move quietly about the ward while receiving the infusions.

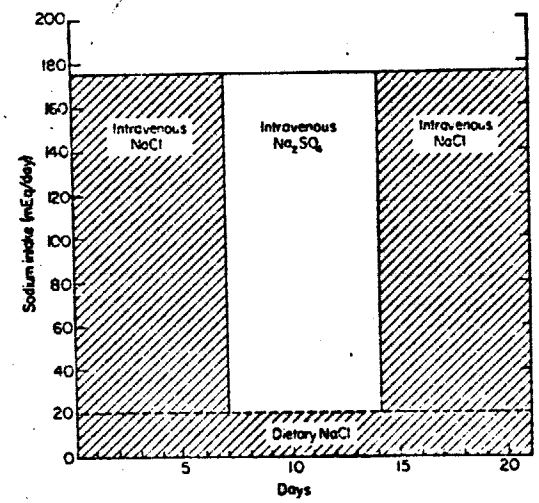

Fig. 1. Diagram of experimental protocol: A constant diet containing approximately 20 mEq NaCl/day was given throughout each balance study. During the first week 155 mEq of NaCl was infused over 8 hr each day. During the second week 155 mEq of Na_2SO_4 was infused over 8 hr each day. In four of the six studies employing this protocol, the balance was continued for one to three additional weeks of alternating NaCl and Na_2SO_4 infusions.

Dietary sodium (9–13 mEq/day), chloride (16–20 mEq/day) and potassium (80–90 mEq/day) were determined on two occasions during each balance study. An entire day's diet was homogenized, and analyses were performed in duplicate.

Venous blood for measurement of pH was drawn with minimal stasis after the subject had been at rest for a least 15 min. The heparin-rinsed, blood-filled syringe was capped and placed in ice until the determination was performed. Venous blood for total CO_2 determination was drawn under mineral oil and handled anaerobically. Twenty-four-hour urine samples were kept refrigerated in bottles containing thymol, phenyl mercuric nitrate and mineral oil.

Analytical methods have been described previously (Lemann & Relman, 1959; Lennon, Lemann & Relman, 1962).

TABLE 1. Serum electrolyte and urine electrolyte and acid excretion in a normal subject alternately infused with NaCl and Na_2SO_4

			Plasma				Urine									
Day	Body weight (kg)	Blood pH	Total CO_2 (m-moles/l)	Na^+ (mEq/l)	K^+ (mEq/l)	Cl^- (mEq/l)	pH	HCO_3^- (mEq/day)	TA (mEq/day)	NH_4^+ (mEq/day)	'Net acid' (mEq/day)	Na^+ (mEq/day)	K^+ (mEq/day)	Cl^- (mEq/day)	$SO_4^=$ (mEq/day)	Organic acid (mEq/day)
1*	67·2	7·41	26·8	143	4·2	108	6·58	15·3	19·8	41·6	45·6	86·8	99·9	105	39·5	73·7
2*	67·1						6·49	12·7	20·5	41·3	49·1	133	84·3	145	36·3	70·6
3*	67·1	7·40	27·7	142	4·1	107	6·45	13·5	25·1	44·0	55·6	185	79·3	186	38·2	60·1
4*	67·1						6·28	7·35	26·3	46·0	65·0	165	62·8	160	36·3	77·1
5*	67·1						6·40	9·51	21·0	40·6	52·1	167	61·9	165	31·3	68·1
6*	67·1						6·40	11·8	24·8	43·6	56·6	204	76·7	198	33·6	77·8
7*	66·8	7·40	28·1	145	4·1	109	6·40	10·4	25·8	43·8	59·2	181	80·2	176	31·1	75·3
8†	67·0	7·41	25·9	141	4·2	108	6·10	4·66	28·5	47·2	71·0	175	75·2	55·3	181	81·9
9†	66·8						6·10	3·44	26·1	43·8	66·5	133	74·6	24·3	162	72·6
10†	66·7	7·44	29·2	141	4·3	106	6·30	8·11	24·8	40·6	57·3	155	75·5	25·8	177	83·1
11†	66·8						6·40	3·40	24·9	38·5	50·0	182	92·3	25·1	180	83·7
12†	66·2						6·50	15·3	25·8	44·2	54·7	175	84·5	26·2	188	79·9
13†	66·3	7·43	27·4	141	4·4	106	6·28	7·14	27·3	41·2	61·4	148	73·0	19·1	174	80·8
14*	66·6	7·39	27·2	140	4·2	108	6·58	16·5	25·9	35·8	45·2	84	73·6	70·7	34·9	73·6
15*	66·6						6·48	13·0	24·7	37·2	48·9	107	74·0	115	32·4	79·4
16*	66·1	7·42	27·3	141	4·1	110	6·58	19·1	28·8	45·8	55·5	214	89·4	225	36·7	84·4
17*	66·2	7·42	27·3	142	4·2		6·45	12·3	25·4	45·1	58·2	175	75·4	191	33·5	81·3

* 155 mEq of NaCl i.v. daily.
† 155 mEq of Na_2SO_4 i.v. daily.

TABLE 2. Serum electrolyte and urine electrolyte and acid excretion during alternate periods of infusion with NaCl and Na_2SO_4 in a normal subject given methionine

			Plasma				Urine								
Day	Body weight (kg)	Blood pH	Total CO_2 (m-moles/l)	Na^+ (mEq/l)	K^+ (mEq/l)	Cl^- (mEq/l)	pH	TA (mEq/day)	NH_4^+ (mEq/day)	'Net acid' (mEq/day)	Na^+ (mEq/day)	K^+ (mEq/day)	Cl^- (mEq/day)	$SO_4^=$ (mEq/day)	Organic acid (mEq/day)
0	66·0	7·41	27·7	142	4·4	110									
1*	66·0						5·41	38·7	67·4	106	151	66·2	172	81·9	69·2
2*	66·1						5·58	33·6	62·6	96·2	156	70·4	174	85·1	69·2
3*	66·0	7·43	27·5	141	4·4	110	5·35	39·9	71·2	111	168	69·0	195	87·9	72·4
4*	66·2						5·30	39·9	67·2	107	133	59·0	148	86·5	67·4
5*	66·1	7·41	27·1	140	4·4	110	5·40	42·9	72·8	116	166	64·3	185	95·3	68·8
6*	66·1						5·30	42·4	75·5	118	166	67·5	186	91·3	72·4
7*	66·2	7·43	27·1	142	4·4	112	5·45	34·4	67·7	102	159	62·8	174	80·9	65·0
8†	66·1	7·41	29·9	141	4·5	108	5·21	38·1	69·6	108	180	68·8	65·8	231	74·3
9†	66·0						5·15	44·9	74·5	119	157	64·9	36·9	239	73·2
10†	66·0	7·41	28·9	142	4·3	106	5·22	45·0	72·7	118	157	60·2	26·7	243	68·3
11†	66·0						5·30	43·5	69·3	113	157	68·7	25·6	236	73·3
12†	66·1	7·41	27·8	142	4·1	100	5·20	43·1	70·2	113	145	66·2	17·4	237	70·8
13†	66·2						5·05	43·9	71·5	115	141	63·8	13·6	227	71·0
14†	66·1	7·42	28·2	146	4·1	108	5·20	43·4	70·5	114	142	63·9	12·7	235	71·2
15*	66·8	7·42	26·8	146	4·1	110	5·50	39·4	65·0	104	79·2	51·8	134	89·3	64·1
16*	66·4						5·59	36·9	67·2	104	189	67·5	185	85·0	65·2
17*	66·4	7·40	26·1	147	4·7	111	5·35	36·7	69·4	106	191	57·7	185	102	64·0
18*	66·6						5·35	39·9	76·5	116	193	75·5	206	105	74·9
19*	66·4	7·41	26·3	146	4·5	106	5·40	42·8	73·0	116	165	71·1	182	92·1	72·0
20*	66·6						5·50	36·4	61·8	102	164	63·4	150	85·3	66·2
21*	66·6	7·40	27·7	143	3·9	110	5·43	37·0	62·2	99·2	166	60·8	164	90·4	67·2

* 155 mEq of NaCl i.v. daily.
† 155 mEq of Na_2SO_4 i.v. daily.

RESULTS

Balances with 8-hr infusions

As expected, acid excretion was higher in the two studies in which methionine was given. However, the results were otherwise the same and therefore the data from all of the balances with 8-hr infusions will be presented together.

The results of representative balance studies are shown in Tables 1 and 2 and Figs. 2 and 3.

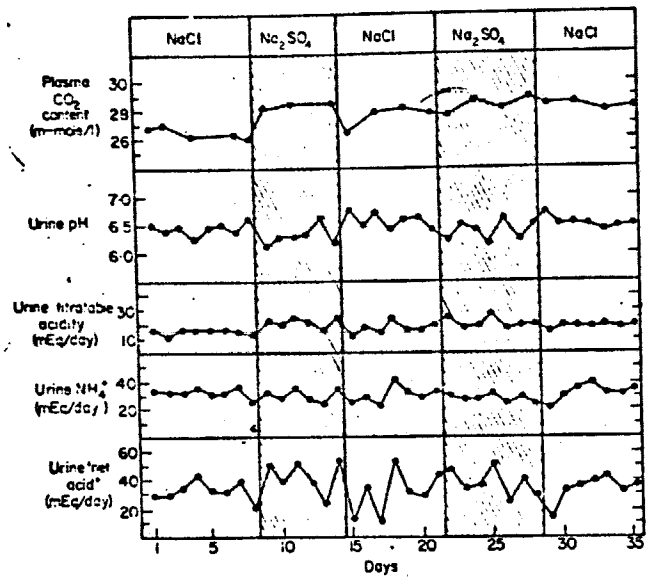

FIG. 2. Plasma CO_2 content, urine pH, and urinary excretion of TA, NH_4^+, and 'net acid' in a subject during alternate periods of NaCl and Na_2SO_4 infusion (8 hr/day).

Subjects depicted in Fig. 3 and Table 2 received 6 g/day of supplemental methionine. The essential data from all six balances with 8-hr infusions are summarized in Table 3, which gives the means and standard deviations of the values obtained during the periods of infusion with sodium chloride or sodium sulphate.

Urine pH. Urine pH fell by 0·05–0·48 units on the 1st day of each sulphate period, and rose by 0·23–0·84 units each time saline was reinstituted (Tables 1 and 2 and Figs. 2 and 3). However, these changes in urine pH were usually not sustained beyond the 1st or 2nd day. As shown in Table 3, mean urine pH was lower during the sulphate periods in each subject than during the chloride periods. In four patients the P value for this difference was 0·05 or less; in the other two the difference was not significant. When all the pH values during sulphate infusion periods were pooled and compared by the paired t-test with those during chloride infusion this difference was not significant.

Titratable acid (TA). As would be expected from the change in urine pH, titratable acid excretion rose slightly at the start of each sulphate infusion period and fell slightly each time saline was reinstituted. The initial changes were 1–12 mEq/day and were usually not sustained beyond the first 2 or 3 days of each period (Tables 1 and 2 and Figs. 2 and 3). During the periods of sulphate infusion, the mean excretion of titratable acid for all six studies was 4·0 mEq/day greater than during the periods of chloride infusion, and this small difference was significant by the paired t-test ($P<0·01$). However, when analysed individually the differences

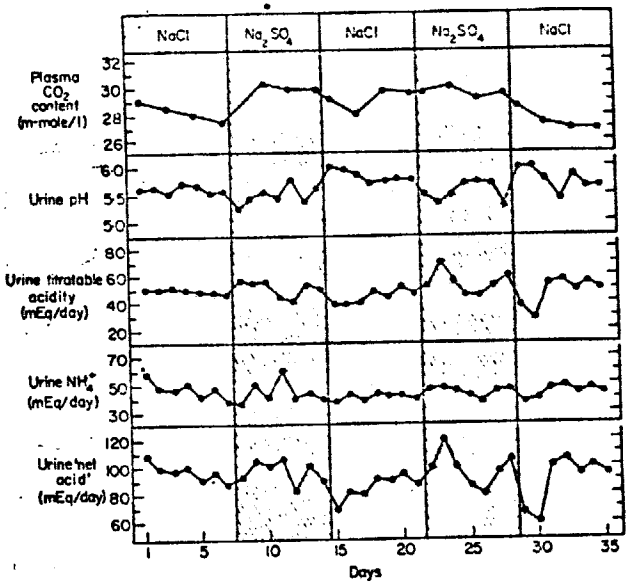

FIG. 3. Plasma CO_2 content, urine pH, and urinary excretion of TA, NH_4^+, and 'net acid' in a subject whose diet was supplemented with 6 g of methionine daily to increase the acid load. The protocol is otherwise the same as that shown in Fig. 2.

in titratable acid between sulphate and saline periods were significant in only half of the studies ($P<0·05$).

Ammonium. There was no consistent or significant change in ammonium excretion when sulphate was started (Tables 1 and 2 and Figs. 2 and 3). The mean excretion of ammonium during sulphate periods was essentially the same as that during chloride periods, whether the studies were considered as a group or analysed individually (Table 3).

Net acid. In general net acid tended to be more variable than the other variables of acid excretion (Figs. 2 and 3). Net acid excretion increased by 4–31 mEq on the first day of sulphate infusion and decreased by 10–42 mEq when chloride infusions were reinstituted (Tables 1 and 2 and Figs. 2 and 3). These changes were sometimes sustained throughout the period of infusion but in other instances disappeared after several days. The difference between mean acid excretion during sulphate and that during chloride was significant ($P<0·05$) in only one of the

TABLE 3. Mean (±SD) plasma CO_2 content, urinary pH and daily excretion of titratable acid (TA), ammonium (NH_4^+) and 'net acid' in normal subjects during alternate periods of intravenous NaCl and Na_2SO_4*

Subject	NaCl	Na_2SO_4	NaCl	Na_2SO_4	NaCl
W.F.					
Plasma CO_2 content	28·7 ±0·5	27·0 ± 1·4			
Urine pH†	6·59+0·08 −0·07	6·47+ 0·04 − 0·04			
Urine TA	13·0 ±1·70	16·0 ± 1·44			
Urine NH_4^+	32·2 ±4·82	29·5 ± 3·77			
Urine 'net acid'	26·0 ±7·29	31·5 ± 2·82			
F.A.					
Plasma CO_2 content	28·3 ±0·5	29·3 ± 1·3			
Urine pH	6·12+0·08 −0·07	5·89+ 0·27 − 0·17			
Urine TA	22·4 ±1·82	26·9 ± 3·65			
Urine HN_4^+	24·6 ±1·92	25·4 ± 3·38			
Urine 'net acid'	41·1 ±3·91	49·7 ± 9·16			
W.F.					
Plasma CO_2 content	27·5 ±0·7	27·5 ± 1·6	27·3 ± 0·1		
Urine pH	6·42+0·10 −0·08	6·26+ 0·19 − 0·14	6·52 + 0·07 − 0·06		
Urine TA	23·3 ±2·77	26·2 ± 1·44	26 2 ± 1·80		
Urine NH_4^+	43·0 ±1·90	42·6 ± 3·10	41·0 ± 5·22		
Urine 'net acid'	54·7 ±6·47	60·1 ± 7·75	51·9 ± 5·96		
J.S.					
Plasma CO_2 content	26·4 ±0·4	28·3 ± 0·2	27·6 ± 0·8	28·4 ± 0·6	28·2 ± 0·3
Urine pH	6·43+0·14 −0·11	6·29+ 0·19 − 0·14	6·55+ 0·17 − 0·12	6·33+ 0·22 − 0·14	6·52+ 0·10 − 0·08
Urine TA	13·8 ±1·92	21·3 ± 4 02	17·0 ± 3·99	20·1 ± 3·65	17·4 ± 1·67
Urine NH_4^+	32·2 ±2·78	30·5 ± 4·54	30·1 ± 5·84	27·4 ± 2·72	31·5 ± 5·06
Urine 'net acid'	32·8 ±7·11	43·2 ±11·6	30·7 ±15·9	37·7 ±10·0	33·2 ± 9·46
J.S.‡					
Plasma CO_2 content	27·2 ±0·4	28·7 ± 0·9	26·7 ± 0·7	28·6 ± 0·3	27·3 ± 0·4
Urine pH	5·39+0·10 −0·08	5·18+ 0·09 − 0·07	5·44+ 0·10 − 0·08	5·26+ 0·11 − 0·09	5·59+ 0·20 − 0·14
Urine TA	38·8 ±3·62	43·1 ± 2·34	38·7 ± 2·11	39·5 ± 4·07	36·4 ± 3·23
Urine NH_4^+	69·1 ±4·27	71·1 ± 1·87	68·1 ± 5·09	68·4 ± 8·04	66·9 ± 3·58
Urine 'net acid'	108 ±7·67	114 ± 3·64	107 ± 6·44	108 ±11·5	103 ± 7·52
J.G.‡					
Plasma CO_2 content	28·2 ±0·7	29·6 ± 0·8	29·1 ± 0·8	29·6 ± 0·4	27·4 ± 0·8
Urine pH	5·62+0·08 −0·06	5·48+ 0·21 − 0·15	5·83+ 0·13 − 0·10	5·51+ 0·24 − 0·15	5·72+ 0·41 − 0·21
Urine TA	49·8 ±1·17	52·0 ± 6·37	44·9 ± 5·51	54·7 ± 9·70	46·9 ±11·5
Urine NH_4^+	46·0 ±5·05	44·6 ± 8·41	40·9 ± 2·37	44·1 ± 3·93	44·7 ± 4·21
Urine 'net acid'	96·5 ±7·10	96·6 ± 8·88	85·1 ± 8·75	98·9 ±13·0	89·4 ±19·3

* CO_2 contents are expressed in m-moles/l, and TA, NH_4^+ and 'net acid' in mEq/day.
† Mean pH (±SD) was calculated by converting individual values to H^+.
‡ Received 6 g of methionine orally throughout period of study.

six subjects. However, when the group data were pooled, net acid excretion was on the average 6·8 mEq/day higher during sulphate than during chloride periods and the difference between periods was significant ($P<0·01$).

Electrolyte excretion. Daily sodium excretion varied considerably, although the mean rate of excretion was not significantly different during sulphate as compared with chloride periods. In some studies a small negative balance of sodium occurred on the 1st day of sulphate administration and a positive balance on the 1st day of reinstituting chloride, but subjects were always in approximate sodium balance over each period as a whole.

Chloride and sulphate excretion paralleled intake. By the end of each sulphate period urine chloride ranged from 13 to 25 mEq/day. Potassium excretion was essentially constant throughout each study.

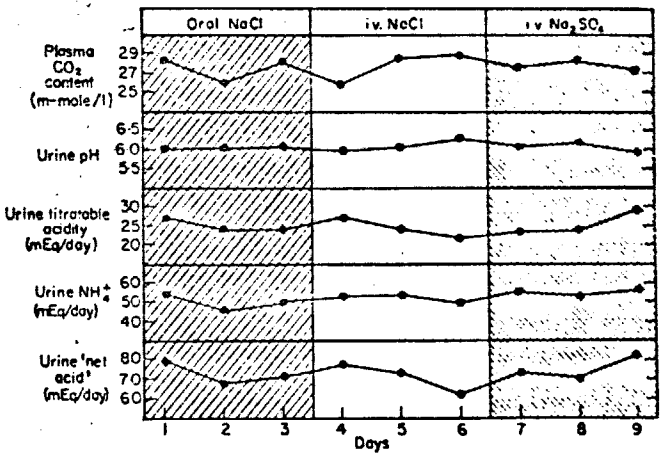

FIG. 4. Plasma CO_2 content, urine pH, and urinary excretion of TA, NH_4^+, and 'net acid' in a subject given supplemental oral NaCl, 155 mEq/day, for 3 days and thereafter infused continuously for 3 days with NaCl, 155 mEq/day, and for a final 3 days with Na_2SO_4, 155 mEq/day.

Blood composition. Plasma CO_2 content sometimes rose slightly when sulphate was started and sometimes fell slightly when chloride was reinstituted (Tables 1 and 2, Figs. 2 and 3). In five of the six studies, the mean CO_2 content during sulphate was 0·1–1·6 m-moles/l higher than during chloride periods, but this difference was significant ($P<0·02$) in only three. When the mean CO_2 content for all sulphate periods was compared with that during chloride, the difference was only 0·7 m-moles/l, and was not significant.

Blood pH, and plasma concentrations of sodium, potassium and chloride were not significantly or consistently affected by the sulphate infusions.

Balances with continuous infusions

The results of one of the two balance studies in which chloride and sulphate infusions were given continuously for 3 days each are shown in Fig. 4. Net acid excretion averaged 3 mEq/day

higher during the sulphate infusion than during chloride infusion. In the other study, not shown here, net acid excretion increased by 12 mEq/day. In neither study did plasma CO_2 content or blood pH vary significantly.

DISCUSSION

Our results demonstrated only very slight and transient increases in urinary acidity when the anion load was changed from largely chloride to largely sulphate. Net acid excretion did not change significantly in most subjects, but there was on the average a small increase when the data from all subjects were pooled. This difference, although of statistical significance, was negligible when compared to the large change in the load of non-reabsorbable anion, and was not sufficient to cause a significant increase in plasma bicarbonate concentration. Although in several experiments there was a slight tendency for plasma CO_2 content to rise with sulphate loading and to fall with the re-institution of chloride, these changes were not consistent, nor were they of statistical significance. We conclude therefore that the nature of the anion load does not play an important role in renal acid–base regulation under the conditions of these experiments.

The design of these experiments permitted us to exchange most of the chloride intake for sulphate, without changing the normal sodium load or affecting endogenous acid production. Our intention was to mimic the endogenous production of sulphate by slowly infusing the anion load intravenously. For practical reasons the infusions usually were given for only 8 hr each day. This raised the possibility that significant increases in plasma bicarbonate and urine acid excretion during or immediately following the sulphate infusions might be compensated for by reverse changes during the second half of each 24-hr balance period. However, we ruled this out in a few studies by collecting 12-hourly urine specimens and by checking plasma CO_2 content at several times during the day. These observations disclosed no evidence of significant transient effects caused by the infusion. Further convincing evidence that the timing of the infusions was not influencing the results was provided by the two studies in which we did manage to administer the sulphate and chloride loads as continuous 24-hr infusions. The data from these two experiments were essentially the same as those obtained in the studies with shorter infusions.

It must be remembered that in these studies chloride was never completely removed from the diet, so that during the periods of sulphate infusion there were always at least 12 mEq chloride/day in the urine. What might have occurred had chloride been totally removed from the diet, or had the subjects been depleted of sodium or given sodium-retaining steroids before the infusion of the sulphate, remains to be determined. In two preliminary experiments (unpublished data) we administered 150 mEq of isotonic sodium sulphate intravenously for 8 hr each day for 1 week to normal subjects maintained on a diet virtually free of sodium and chloride. In one subject there was a significant but transient increase in daily acid excretion and a sustained elevation of plasma bicarbonate concentration during the period of sulphate infusion. In the other subject there was no significant effect. More studies will obviously be required to settle this point, but regardless of the outcome it is clear that results which depend on the deprivation of sodium and chloride cannot be used to explain the regulation of acid excretion under ordinary circumstances. We believe that the experimental protocol used in the experiments described

in this paper most nearly represents the normal circumstances under which renal homeostasis of acid–base balance takes place.

It must be remembered that the administration of sulphate in the present experiments was not accompanied by any increase in the sodium load. Our studies differ in this respect from the acute experiments in man reported earlier by Schwartz *et al.* (1955) and from the recent experiments in dogs reported by Berkman *et al.* (1967). In both of these studies the intravenous sulphate load was administered rapidly together with an equivalent quantity of additional sodium. In the human experiments (Schwartz *et al.*, 1955) the sulphate infusion increased acid excretion in subjects previously stimulated to reabsorb sodium, but not in those who were in normal sodium balance. In the dogs (Berkman *et al.*, 1967) even a sodium-free diet did not result in increased acid excretion or a rise in plasma bicarbonate concentration after the sulphate infusion. Presumably the difference between these results could be due to the fact that the relatively larger sodium load in the dog stimulated mechanisms for sodium diuresis that nullified the effect of the prior period of sodium deprivation. Regardless of this difference, however, the results of these acute experiments in both man and dog tend to support our present conclusions by showing that the reabsorbability of the anion load is not by itself a primary and overriding determinant of acid excretion.

If the excretion of acid in response to the normal endogenous acid load is not dependent upon the reabsorbability of the endogenous anions, it presumably must be related in some way to the protons that are released *pari passu* with the anions. However, considerable fluctuation in daily acid excretion occurs without perceptible antecedent changes in plasma pH and bicarbonate. Furthermore, it is unlikely that the relatively modest normal variations in acid load would produce changes in the intracellular acidity of the renal tubules. It therefore seems reasonable to assume that it is not the acidifying effect of the protons *per se* that is responsible for the stimulation of acid excretion. It appears to us more likely that the normal regulation of acid excretion results from the interplay of the several independent mechanisms regulating the excretion of fixed cations and anions. Thus, the administration, or endogenous production, of any acid creates an anion excretory load without providing equivalent fixed cation. Excretion of the anion and conservation of fixed cation requires the secretion of appropriate quantities of hydrogen ion by the tubule. The immediate stimulus for this secretion is presumably a variation in transtubular potential. It is therefore to be expected that hydrogen ion balance would be regulated with approximately the same precision as the balance of sodium, potassium and other fixed cations, and this prediction would appear to be valid (Sartorius, Roemmelt & Pitts, 1949; Relman, Lennon & Lemann, 1961).

We suggest that the mechanism proposed above accounts for only one facet of the renal regulation of acid balance. Another important aspect is the regulation of the bicarbonate threshold. Although it is clear that the latter can be influenced under certain extreme conditions by the availability of reabsorbable anion, other factors such as P_{CO_2} (Brazeau & Gilman, 1953; Relman, Etsten & Schwartz, 1953; Dorman, Sullivan & Pitts, 1954) and tubular cell electrolyte composition (Fuller, Macleod & Pitts, 1955; Rector, Bloomer & Seldin, 1964; Lambie, Relman & Schwartz, 1959) are probably more frequently of importance. In any event, the level at which the bicarbonate threshold is set will determine the steady state concentration of plasma bicarbonate around which the mechanisms responsible for external acid balance will operate. Whenever the acid load is sufficient to lower the plasma bicarbonate concentration below the threshold level, the urine will become more acid and the urinary buffers will be more

fully saturated with hydrogen ion, thereby facilitating the net excretion of acid. Persistent acidaemia probably brings another homeostatic mechanism into play by stimulating renal tubular synthesis of ammonia (Goodman, Fuisz & Cahill, 1966; Goorno, Rector & Seldin, 1967).

In summary, our view of the normal regulation of acid excretion assigns no critical role to the nature of the anion load or to the quantitative acidifying effects of the protons. We propose instead that acid-base regulation results from the confluence of independent mechanisms that regulate the bicarbonate threshold, the excretion of fixed anions and cations, and the synthesis of ammonia.

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THE CARBOXYLASE ENZYME SYSTEM*

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(Received for publication, June 15, 1938)

Carboxylase was discovered by Neuberg and Hildesheimer in 1911 (1). It is a highly specific enzyme as it only attacks α-keto acids, such as pyruvic acid, converting them into the corresponding lower aldehydes; *e.g.*, pyruvic acid into acetaldehyde. The coenzyme dependency of carboxylase, however, was only noticed 21 years later by Auhagen (2). This investigator has shown that when dry yeast was washed with Na_2HPO_4 it lost its power to decarboxylate pyruvic acid and on the addition of cocarboxylase, which he obtained in a semipure state, the enzyme became active again. To restore full activity, however, magnesium ions appeared to be necessary. This was known to be the case with zymase.

Recently Lohmann and Schuster (3) have isolated cocarboxylose from bottom yeast in a pure state and found that it is the pyrophosphoric acid ester of thiamine. In support of their work it had been shown that dry yeast (4), washed dry yeast (5), and duodenal phosphatese (5, 6) convert thiamine into cocarboxylase. Peters (7) found that "vitamin B_1 is phosphorylated rapidly under the conditions of a catatorulin test." Stern and Hofer (8) attempted to convert thiamine into its pyrophosphoric acid ester by treatment with phosphorus oxychloride. While this reagent was very useful in the conversion of lactoflavin (vitamin B_2) into flavin monophosphoric ester (9), which is one of the coenzymes of the yellow oxidation system of Warburg and Christian, only a small amount of the thiamine could be phosphorylated by $POCl_3$. Apparently $POCl_3$ is not an efficient reagent for the

* Preliminary reports of part of this paper have appeared (*J. Am. Chem. Soc.*, 60, 730 (1938); *Proc. Soc. Exp. Biol. and Med.*, 38, 888, 889 (1938)).

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introduction of the pyrophosphate group. Nevertheless, Stern and Hofer have shown by cataphoretic tests that conversion of the vitamin to its diphosphoric ester took place.

In the present paper a synthesis will be described by which thiamine may be completely converted into cocarboxylase. The coenzyme has been obtained in crystalline form. A series of new activators of the carboxylase-cocarboxylase system has been found and a specific function of cocarboxylase has been observed. Experiments will be presented showing that, similar to thiamine, cocarboxylase is also a growth-promoting substance.

EXPERIMENTAL

Synthesis and Purification[1] *of Cocarboxylase*—500 mg. of sodium pyrophosphate are placed in a Pyrex test-tube and heated until all of the water of crystallization is removed. 1 cc. of orthophosphoric acid (C.P. 85 per cent) is placed in another large Pyrex test-tube and heated until a slight amount of solid deposit forms on the side of the tube. Then the pyrophosphate is added and the mixture gently heated until solution takes place. After a few minutes of cooling 500 mg. of thiamine are added and the contents of the tube are well mixed. The tube is placed in an oil bath of 155°, kept there for 15 minutes, and constantly stirred. Then the tube is removed and, after cooling, the solid mass is dissolved in 10 cc. of cold water. Cold saturated $Ba(OH)_2$ solution is added until no more precipitate forms and the solution is just commencing to turn yellow. (The thiochrome compound of cocarboxylase is inactive. It becomes active again, however, on acidification.) The precipitate is centrifuged off and the supernatant fluid is decanted. The precipitate is extracted three times with 50 cc. of cold water. All four supernatant fluids are united and, after cooling, 3 per cent H_2SO_4 is added to slight blue reaction of Congo red paper. The $BaSO_4$ is centrifuged off and discarded. The Ba-free solution is concentrated to 30 cc. in a vacuum at 25°. It is cooled in ice water and 15 to 20 volumes of a mixture

[1] In the purification and identification of synthetic cocarboxylase Mr. J. Weijlard of the Research Laboratory of Merck and Company, Inc., has collaborated. While this paper was in press the cocarboxylase was obtained in a 100 per cent pure state. Hydrolysis and cleavage products were analyzed. The results of this work will be published elsewhere.

of 1 part of absolute alcohol and 2 parts of ether is added which precipitates the thiamine pyrophosphate in the form of microscopic needles. Sometimes a gummy mass forms which will turn into long macroscopic needles on short standing in the cold. The product obtained on six recrystallizations from the alcohol-ether mixture, redissolved each time in 10 cc. of 0.1 N HCl and dried in a vacuum over H_2SO_4, is readily soluble in water and free of inorganic salts.

Activity and General Properties—This synthetic preparation is practically as active as natural cocarboxylase (very kindly furnished by Professor Lohmann). Phosphorus and thiamine content, however, indicates that my cocarboxylase still contains a small amount of impurities.

Similar to the natural cocarboxylase, the synthetic coenzyme becomes inactive on 15 minutes boiling with N HCl, and the total phosphorus is liberated only with difficulty in about 5 hours at 100°. Kidney phosphatase also hydrolyzes the synthetic coenzyme. In this respect it resembles the cocarboxylase obtained by enzymic synthesis (6).

Cocarboxylase forms the theoretical amount of thiamine when treated by an alkaline ferricyanide solution. The ferrocyanide formed during the reaction may be converted to Prussian blue and measured colorimetrically (10).

Cocarboxylase (10 micrograms) gives a yellow color with the formaldehyde-azo test (Kinnersley-Peters (11)). Thiamine gives a red color.

New Activators of the Carboxylase-Cocarboxylase System—Recently Lohmann and Schuster (3) have shown that manganese ions activate the carboxylase-cocarboxylase enzyme system better than magnesium ions. Copper, iron, bismuth, zinc, and cadmium salts as well as sodium flouride inhibit the activity of the enzyme carboxylase (12). It is well known that certain amylases (13) cannot hydrolyze starch in the absence of certain salts. Their activation, however, is not limited to a few ions. One might expect that with carboxylase similar conditions exist. My expectations were correct. Several neutral salts such as Na_2SO_4, NaCl, KCl, as well as NaCN, were found to activate the carboxylase-cocarboxylase system (see Table I). The fact that NaCN activates this system shows that carboxylase is not a heavy metal-

containing enzyme. It activates probably in part because it forms a more reactive enol compound (cyanohydrin) with pyruvic acid. NaCN is a good activator, since only one-tenth the concentration is required to obtain the activation similar to that shown by the neutral salts. While Mn ions activate best, the order of activation obtained by NaCN is close to the one obtained by $MgCl_2$. Li_2SO_4 activates slightly and in dilute concentration only. $NaNO_3$ does not activate. As in the case of amylases, no explanation can be given of why these salts activate the carboxylase-cocarboxylase system.

TABLE I

Activation of Carboxylase-Cocarboxylase System by Various Salts

All samples contained 30 micrograms of cocarboxylase except in Experiment 10.

In all experiments described in this paper synthetic cocarboxylase and natural cocarboxylase were employed with identical results.

Experiment No.	Salt employed	Carbon dioxide		
		10 min.	20 min.	30 min.
		c.mm.	c.mm.	c.mm.
1	$MgCl_2$ (0.1 mg. Mg)	53	88	112
2	$MnSO_4$ (10 micrograms Mn)	51	78	98
3	Na_2SO_4 (4 mg. Na)	38	64	81
4	NaCl (4 mg. Na)	37	62	79
5	KCl (4 mg. K)	36	62	77
6	NaCN (0.4 mg. Na)	38	63	78
7	Li_2SO_4 (0.4 " Li)	25	44	59
8	" (4 mg. Li)	20	35	47
9	No salt	18	34	46
10	" " no cocarboxylase	0	0	0

While it is not difficult to remove all of the cocarboxylase from the dry yeast (Table I, Experiment 10), it is not possible by the extensive washing applied to remove all of the other activators of carboxylase (Table I, Experiment 9), and some activation is apparently caused by the buffer (phosphate). In this experiment acid- and alkali-washed dry yeast was employed (see below).

Specific Function of Cocarboxylase—It is believed that cocarboxylase combines with the inactive enzyme carboxylase to form a new compound which in the presence of magnesium ions becomes

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Carbon dioxide		
10 min.	20 min.	30 min.
c.mm.	c.mm.	c.mm.
53	88	112
51	78	98
38	64	81
37	62	79
36	62	77
38	63	78
25	44	59
20	35	47
18	34	46
0	0	0

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highly active. This compound, however, has not yet been isolated. Nor has the enzyme carboxylase been obtained in pure state. In any case nothing is known about the nature or mechanism of "activation" of carboxylase by cocarboxylase. The following experiments will show that one of the functions of cocarboxylase is to protect carboxylase, a very labile enzyme, from destruction.

Experiment 1—1 cc. of yeast suspension and 30 micrograms of cocarboxylase in 1 cc. of phosphate of pH 6.2 were placed in the main compartment of a Warburg vessel (17 cc. capacity and two side arms). In one side arm 0.5 cc. of sodium pyruvate (5 mg. of pyruvic acid containing 0.1 mg. of magnesium as $MgCl_2$) was placed. The vessel was connected with a Warburg-Barcroft

TABLE II

Showing Protective Function of Cocarboxylase

Experiment No.	Content of main compartment during first 120 min.	Carbon dioxide (after addition of contents in side arm)		
		10 min.	20 min.	40 min.
		c.mm.	c.mm.	c.mm.
1	Yeast suspension and cocarboxylase	40	66	102
2	" " alone	4	12	24
3	" " and thiamine	5	12	24
4	" " " water	0	0	0

respirometer and shaken for 105 minutes at 30°. Then the stopcock was closed and after 15 minutes of further shaking the pyruvate was washed in from the side arm.

Experiment 2—In another vessel the pyruvate was placed in one side arm and the cocarboxylase was placed in the second side arm, while the main compartment of the vessel contained 1 cc. of yeast suspension. This vessel also was shaken for 120 minutes and then the pyruvate and cocarboxylase were added. Cocarboxylase solutions are very stable between pH 4 and 10 at 30°.

Experiment 3—Here 30 micrograms of thiamine (Merck) in 0.1 cc. of phosphate of pH 6.2 and 1 cc. of yeast suspension were placed in the main compartment of the Warburg vessel. The cocarboxylase and pyruvate were added at the end of 120 minutes.

Experiment 4—The content of this vessel was similar to that

in Experiment 1. Cocarboxylase, however, was replaced by 1 cc. of H_2O.

It may be seen from Experiment 1, Table II, that when cocarboxylase was immediately added to freshly washed yeast and kept at 30° for 120 minutes in the presence of air CO_2 formed after the addition of pyruvate very rapidly. If the washed yeast, however, was kept for the same length of time without cocarboxylase, the enzyme carboxylase lost almost all of its activity and there was hardly any CO_2 formed (Experiment 2). Thiamine had no protective action on carboxylase and no cocarboxylase was formed during the duration of the experiment from the vitamin (Experiment 3). The yeast suspension without added cocarboxylase did not form CO_2 from sodium pyruvate (Experiment 4). Other experiments (not included in Table I) have shown that the protective function cannot be replaced by Ba-adenosinetriphosphate, Mg-hexosediphosphate, or by reduced glutathione.

In these experiments brewers' yeast which had been washed with alkaline phosphate was employed (see below).

Removal of Activators and Cocarboxylase from Dry Yeast. Alkaline Washing—For the cocarboxylase test and for the experiments on the "Specific function of cocarboxylase" brewers' bottom yeast[2] was extensively washed with water and dried at room temperature with the aid of a fan. The alkaline washing was carried out according to Lohmann and Schuster (3). To 2 gm. of dry yeast in a 250 cc. centrifuge flask 100 cc. of 0.1 M Na_2HPO_4 at 30° was added and shaken in a shaking machine for 12 minutes. Then the mixture was centrifuged. The supernatant fluid was discarded. This procedure was repeated once more. The material was washed for 3 minutes with 100 cc. of water at 30°. The washed yeast was suspended in 20 cc. of phosphate of pH 6.2 and was so used.

It is important to note that there is no direct proportionality between cocarboxylase concentration (activity) and CO_2 formation from pyruvate. For instance, if the cocarboxylase concentration is increased 3 times, CO_2 formation is only doubled after the first 15 minutes of the experiment (3, 6, 8). It should also be noted

[2] I am indebted to the Jacob Ruppert Brewery, through the kindness of Mr. E. Muhlhausen, for furnishing the bottom yeast.

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that the carboxylase content of various yeasts differs greatly, and for this reason it is difficult to make exact comparisons of cocarboxylase activities by the enzymic test.

Acid and Alkaline Washing—For the salt activation experiments the dry yeast was washed with acid and alkaline phosphate according to Lohmann and Schuster (Table VII (3)). 2 gm. of dry yeast were washed by shaking in a machine for 10 minutes at room temperature, three times with 100 cc. portions of 0.1 M KH_2PO_4, once with 100 cc. of H_2O, twice with 100 cc. portions of 0.1 M Na_2HPO_4, and once with 100 cc. of H_2O. The washed yeast was suspended in 20 cc. of phosphate, pH 6.2. A solution of sodium pyruvate containing 10 mg. of pyruvic acid per cc. was prepared and adjusted to pH 6.2 with NaOH. No mineral acids were introduced. 0.5 cc. of sodium pyruvate (5 mg.) was placed in the side arm of the Warburg vessels. 1 cc. of washed yeast suspension, 0.5 cc. of cocarboxylase, 0.5 cc. of phosphate of pH 6.2, and 0.5 cc. of salt solution were placed in the main compartment; total volume, 3 cc. The temperature was 28°.

Cocarboxylase Growth Substance—Thiamine has an accelerating action on the growth of yeast. With cocarboxylase acceleration is more pronounced.

In the presence of a proteose-peptone-sucrose-salt medium 1 microgram of the pyrophosphate per cc. increases the growth considerably in 20 hours at 25°. In the following a set of typical experiments is described. To the medium (pH 5.6) containing 5 gm. of sucrose, 1 gm. of proteose-peptone (Difco), 0.3 gm. of KH_2PO_4, and 0.3 gm. of $MgSO_4 \cdot 7H_2O$ in 100 cc. of distilled water, 0.1 mg. of thiamine or 0.1 mg. of thiamine pyrophosphate (in 0.2 cc. of water) was added. The medium was made up fresh every day and was boiled for 10 minutes before use. Controls were run without the vitamin or ester. 100 cc. samples of the medium were placed in large culture dishes and to each 1 cc. of a 0.1 per cent suspension of commercial bakers' yeast (Fleischmann) was added. After 20 hours three of each of the samples were united, centrifuged, and weighed. Those with added thiamine pyrophosphate weighed 5.1 gm.; those with thiamine weighed 4.6 gm.; whereas without the vitamin or coenzyme samples weighed 2.7 gm.

SUMMARY

The synthesis of cocarboxylase (pyrophosphoric acid ester of thiamine) from synthetic thiamine has been described. The synthetic, crystalline coenzyme contains a small amount of impurities. It is, however, as active as the pure natural preparation.

Several salts have been found to be activators of the carboxylase-cocarboxylase system, one of which was NaCN. The fact that NaCN activates the system proves that heavy metals are not a part of this enzyme system. The activation by NaCN is probably due to the formation of a more reactive addition (enol) compound, cyanohydrin, with pyruvic acid.

Experiments have been described which show that cocarboxylase has a specific protective action on carboxylase. The enzyme freed of cocarboxylase is very labile. Cocarboxylase prevents it from rapid inactivation. Adenosinetriphosphate, hexosediphosphate, or reduced glutathione cannot replace cocarboxylase in its protective function.

While in plants thiamine pyrophosphate functions as an indispensable specific coenzyme for carboxylase, in mammalian metabolism it acts as a coenzyme for a pyruvic acid dehydrogenase (14).

Experiments with bakers' yeast show that, similar to thiamine, cocarboxylase also functions as a growth substance, and it appears that the growth-promoting action of thiamine is based on its conversion to cocarboxylase by the living cell. After thiamine has been phosphorylated, it acts as an accelerator of carbohydrate metabolism.

I am grateful to Merck and Company, Inc., and to the Winthrop Chemical Company, Inc., for generous gifts of thiamine, and to Professor Lohmann for a sample of pure (natural) cocarboxylase.

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The Effects of Sulfate on Potassium Transport by Rat Liver Mitochondria*

(Received for publication, January 19, 1967)

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SUMMARY

When rat liver mitochondria were incubated for 15 min at 37° and pH 7.4 in 250 mM sucrose, 50 mM tris(hydroxymethyl)aminomethane hydrochloride, 10 mM $MgCl_2$, 10 mM inorganic orthophosphate, and 1 mM ethylenediaminetetraacetate, the resulting loss of endogenous K^+ was greatly increased by the addition of 2.5 to 10 mM ammonium sulfate or sodium sulfate to the incubation medium. The increased rate of K^+ loss, which could be completely prevented by the addition of ATP, succinate, or glutamate to the medium, began 4 to 8 min after the start of incubation. Ammonium sulfate and sodium sulfate also increased the loss of K^+ when the mitochondria were first suspended at 0° in a solution containing KCl before being added to the incubation medium.

When KCl was added to the mitochondria after 15 min of incubation at 37°, both the passive uptake and the active uptake of K^+ were not affected by the simultaneous addition of either 10 mM ammonium sulfate or sodium sulfate. However, when mitochondria were incubated for 15 min with ammonium sulfate before the addition of KCl, active uptake of K^+ appeared to be abolished.

In the absence of exogenous P_i, sodium sulfate inhibited the loss of endogenous K^+, while ammonium sulfate still caused an increased K^+ loss.

In the absence of exogenous Mg^{++}, both ammonium sulfate and sodium sulfate inhibited the loss of endogenous K^+ whether or not P_i was present in the medium.

Addition of sulfate ions to the incubation medium also increased the leakage from mitochondria of compounds showing an absorption maximum at 259 mμ.

During studies of K^+ transport by isolated rat liver mitochondria, it was found that addition of crystalline yeast hexokinase to the incubation medium caused a marked increase in the loss of endogenous K^+ whether or not glucose also was added. Further studies indicated that small amounts of ammonium sulfate adhering to the crystalline hexokinase caused the increased loss of K^+.

Two groups of investigators have reported on the uptake of sulfate by isolated mitochondria (1, 2). However, these studies were not concerned with the effects of the anion on mitochondrial K^+ movements. The following study shows that sulfate affects both the loss of K^+ from mitochondria and, under certain conditions, the active uptake of K^+ by the mitochondria.

EXPERIMENTAL PROCEDURE

Male albino Sprague-Dawley rats weighing 200 to 300 g and fed Purina laboratory dog chow *ad libitum* were used. Liver mitochondria were isolated at 0–4° from 10% (w/v) homogenates according to the method of Schneider (3), with 175 to 210 ml of the supernatant obtained after removal of the nuclear fraction. The particles were washed twice with a solution containing 250 mM sucrose, 10 mM Tris-HCl (pH 7.4), and 1 mM EDTA-Tris (pH 7.4). The mitochondria were suspended with this solution to a volume of 16 to 19 ml to give a protein concentration of 30 to 45 mg per ml.

When the loss of endogenous K^+ was studied, 0.5 ml of the mitochondrial suspension at 0° was added to a Lusteroid centrifuge tube (approximately 8.5-ml capacity) containing 2.5 ml of medium which had been warmed to 37°. The medium, hereafter referred to as the standard incubation medium, usually contained 184 mM sucrose, 50 mM Tris-Cl (pH 7.4), 1 mM EDTA-Tris (pH 7.4), 12 mM $MgCl_2$, and 12 mM P_i-Tris. Incubations in duplicate were performed with constant shaking (approximately one oscillation per sec) in air at 37° in a Dubnoff metabolic incubator. At the end of the incubation period, the tubes were centrifuged for 3 min at 0° and 18,400 rpm in the No. 29.5 rotor of an International refrigerated centrifuge, model PR-2. The supernatant was discarded, and after the tube was drained for a few minutes, the mitochondrial pellet was quickly rinsed with 6 to 8 ml of the ice-cold solution used for suspending the mitochondria. The tube was again drained, and the inside was dried with filter paper. Two milliliters of water and 2 drops of a 1% (v/v) aqueous solution of Dow-Corning Antifoam B were added to the tube, and the mitochondrial pellet was dispersed with a homogenizing pestle. The resulting mitochondrial sus-

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TABLE I

Effects of ATP and respiratory substrate on increased loss of endogenous K^+ from rat liver mitochondria induced by ammonium sulfate

Rat liver mitochondria were incubated for 15 min at pH 7.4 and 37° in a volume of 3.0 ml. The incubation medium contained 195 mM sucrose (final concentration), 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA-Tris, 10 mM P_i-Tris (when added), and 19.3, 21.9, and 18.3 mg of mitochondrial protein in Experiments 1, 2, and 3, respectively. Additions to the standard incubation medium as shown in the table were made to give the following final concentrations: 30 mM sucrose (in addition to the sucrose already present in the medium), 10 mM ammonium sulfate, 5 mM ATP, and 20 mM substrate. In Experiment 2, P_i was omitted from the incubation medium. In Experiment 3, the medium contained 10 mM P_i, and the additions at 4 and 8 min were made in a volume of 0.1 ml to give a total final volume of 3.1 ml.

Conditions	Mitochondrial K^+ content			
	$-P_i$	$+P_i$	Added to medium before mitochondria	
			30 mM sucrose	10 mM ammonium sulfate
	mμmoles/mg protein			
Experiment 1				
Sucrose, no incubation	84	83		
Sucrose	42	49		
Sucrose + ATP	55	62		
Ammonium sulfate	19	7		
Ammonium sulfate + ATP	69	65		
Experiment 2				
No additions			40	17
ATP			54	74
Succinate			56	43
Glutamate			58	66
α-Ketoglutarate			60	21
β-Hydroxybutyrate			44	14
Experiment 3				
No incubation			80	81
Tris-HCl added at 4 min			47	7
ATP added at 4 min			55	63
Tris-HCl added at 8 min			49	7
ATP added at 8 min			52	35

pension was transferred to a 5-ml graduated cylinder and made up to a volume of 4.0 ml with water. The supension was transferred into a polyethylene tube and stored at −20° until it was to be analyzed for K^+ and protein.

The procedure for studying the uptake of K^+ was similar to that described above except that 0.5 ml of the mitochondrial suspension was added to 2.0 ml (instead of 2.5 ml) of medium. After 15 min of incubation at 37°, 0.5 ml of a solution containing KCl was added to the mitochondrial suspension, and incubation was continued for another 15 min.

K^+ was measured with a Baird-Atomic flame photometer (model DB-4) with lithium nitrate as an internal standard. Protein was determined by the biuret method (4), a standard protein solution being used for the construction of the calibration curve.

The sodium salt of ATP (Sigma), succinic acid (Fisher), L-glutamic acid and α-ketoglutaric acid (Calbiochem), DL-β-hydroxybutyric acid (Mann), and the free acid of EDTA were adjusted to pH 7.4 with Tris. All other reagents used were analytical grade.

When the loss of endogenous K^+ was studied, the notation "no incubation" in the tables means that 0.5 ml of the mitochondrial suspension was added to 2.5 ml of incubation medium, rapidly mixed with a stirring rod, and then immediately centrifuged. Similarly, when the uptake of K^+ was studied, the notation "15-min incubation, add K^+, and centrifuge at once" in the tables means that after the addition of K^+ the suspension was rapidly mixed and then immediately centrifuged.

Leakage from mitochondria of compounds showing an absorption maximum at 259 mμ was determined as follows. Two milliliters of the supernatant obtained after incubation and subsequent centrifugation of the mitochondrial suspension were diluted with 1.5 ml of 12.5% trichloracetic acid. The mixture was centrifuged, and the optical density of the clear solution was determined at 259 mμ.

Each value in both the tables and the figures is the average of duplicate incubations.

RESULTS

Effects of Ammonium Sulfate on Loss of Endogenous K^+—The increased loss of endogenous K^+ induced by ammonium sulfate became apparent at a concentration of 1 to 2.5 mM and reached a maximum at approximately 10 mM. It was greater when P_i was added to the incubation medium than when P_i was omitted.

In order to determine whether the increased loss of endogenous K^+ caused by ammonium sulfate could be prevented by the addition of external sources of metabolic energy, the experiments shown in Table I were performed. In the first experiment, ATP decreased the loss of endogenous K^+ from control mitochondria incubated for 15 min. Similar observations were reported by Christie *et al.* (5). ATP also completely inhibited the increased loss of K^+ induced by 10 mM ammonium sulfate.

When respiratory substrates were added to the incubation medium in the absence of exogenous P_i, succinate, glutamate, and α-ketoglutarate were as effective as ATP in decreasing the loss of endogenous K^+ from control mitochondria (Table I, Experiment 2). β-Hydroxybutyrate did not retard K^+ loss. Of the four substrates tested, only succinate and glutamate prevented the increased loss of K^+ induced by ammonium sulfate.

The action of ammonium sulfate in accelerating the loss of endogenous K^+ took several minutes to become apparent (Fig. 1). When P_i and ammonium sulfate were present in the medium, the loss of K^+ was greatly accelerated between 4 and 8 min after addition of the mitochondria to the incubation medium (Fig. 1, *left*). When P_i was omitted from the medium the onset of accelerated K^+ loss was delayed by about 4 min (Fig. 1, *right*). This would explain the slightly but consistently lower K^+ content of mitochondria incubated for 15 min with 10 mM ammonium sulfate in a P_i-containing medium compared with mitochondria incubated with ammonium sulfate in the absence of P_i (*cf.* Table I, Experiment 1, and Table V).

The increased loss of K^+ could be prevented by adding ATP 4 min after the start of incubation (Table I, Experiment 3). Even when added after 8 min of incubation, ATP partially prevented the increased loss of K^+ induced by ammonium sulfate.

Effect of Temperature—When mitochondria were incubated at 0° instead of 37° in a medium containing P_i, the K^+ content of the mitochondria incubated with 10 mM ammonium sulfate decreased from 95 mμmoles of K^+ per mg of protein at 0 min to 82 mμmoles of K^+ per mg of protein at 120 min. During that same period, the K^+ content of the controls decreased from 85 mμmoles of K^+ per mg of protein to 82 mμmoles of K^+ per mg of protein.

Effects of Ammonium Sulfate on Loss of K^+ from Mitochondria Loaded with KCl—When mitochondria were suspended with a solution containing KCl before being added to the incubation medium, addition of ammonium sulfate to the medium accelerated the loss of K^+ from the mitochondria. The increased K^+ loss was completely prevented by ATP.

Effects of Ammonium Sulfate on Uptake of K^+—Since ammonium sulfate had such a striking effect on increasing the loss of endogenous K^+ from liver mitochondria, it seemed important to determine whether it had any effect on the accumulation of exogenous K^+. The experimental approach, similar to that used by Christie *et al.* (5), was to deplete the mitochondria of endogenous K^+ by incubating them for 15 min at 37°. KCl was then added, and the mitochondria were incubated for another 15 min. The experiment presented in Table II shows that the addition of ammonium sulfate together with KCl had little, if any, effect on the accumulation of K^+. Furthermore, ammonium sulfate had no effect on the increased uptake of K^+ when ATP was added with the KCl.

The experiment shown in Table III was designed to study the effects of ammonium sulfate on the accumulation of K^+ when the mitochondria were incubated for 15 min with 10 mM ammonium sulfate *before* the addition of KCl. Under these conditions, the addition of 10 mM KCl to the ammonium sulfate-incubated mitochondria resulted in a 3- to 4-fold increase in in the K^+ content, which then did not change during the second 15-min incubation period. Addition of ATP with the KCl retarded the loss of K^+ from the control mitochondria but had little, if any, effect on the K^+ content of the mitochondria incubated with ammonium sulfate. Qualitatively similar results were obtained with the addition of either 2 mM KCl or 25 mM KCl (data not presented in detail). These results suggest that preincubation with ammonium sulfate abolishes active uptake of K^+ by mitochondria.

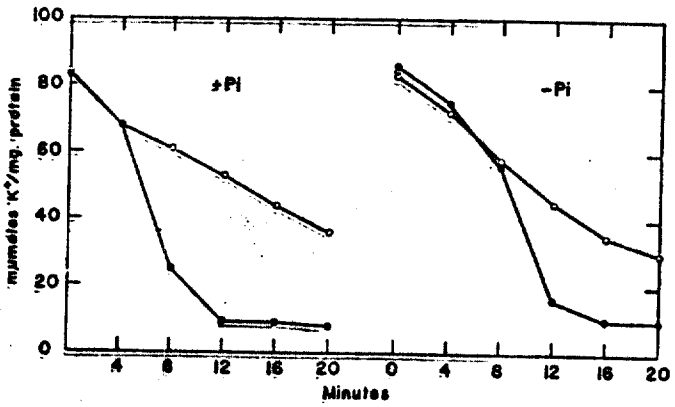

FIG. 1. The effects of ammonium sulfate on the loss of endogenous K^+ from rat liver mitochondria. A mitochondrial suspension (0.5 ml) containing 19.9 mg. of mitochondrial protein ($+P_i$) or 19.2 mg of mitochondrial protein ($-P_i$) was added to 2.5 ml of a medium containing 184 mM sucrose, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA-Tris (pH 7.4), 12 mM $MgCl_2$, 12 mM P_i-Tris (when added), and either 12 mM ammonium sulfate (●) or 36 mM sucrose (○) which was in addition to the sucrose already present in the medium. Incubations were performed at 37° for various periods of time. For other details, see "Experimental Procedure."

TABLE II

Effects of ammonium sulfate on uptake of K^+ by rat liver mitochondria

ATP (5 mM, final concentration) and 10 mM ammonium sulfate or 30 mM sucrose (the latter added in addition to that already in the standard incubation medium) were added simultaneously with 10 mM KCl at the end of the first 15-min incubation period. The incubation medium contained 18.9 mg of mitochondrial protein and, when added, 10 mM P_i. Other conditions were the same as in Table I.

Conditions	Mitochondrial K^+ content			
	$-P_i$		$+P_i$	
	−ATP	+ATP	−ATP	+ATP
	mμmoles/mg protein			
No incubation	81		79	
15-min incubation only	45		50	
15-min incubation, add K^+ + sucrose, and centrifuge at once	71		75	
15-min incubation, add K^+ + sucrose, and incubate 15 min more	52	63	49	71
15-min incubation, add K^+ + ammonium sulfate, and incubate 15 min more	49	70	44	78

TABLE III

Effects of preincubation with ammonium sulfate on uptake of K^+ by rat liver mitochondria

ATP (5 mM, final concentration) was added simultaneously with 10 mM KCl at the end of the first 15-min incubation period; 30 mM sucrose or 10 mM ammonium sulfate was added to the standard incubation medium before the mitochondria. The standard incubation medium contained 10 mM P_i and 18.5 mg of mitochondrial protein. Other conditions were the same as in Table I.

Conditions	Added to medium before mitochondria	
	Sucrose	Ammonium sulfate
	mμmoles K^+/mg protein	
No incubation	83	81
15-min incubation only	51	10
15-min incubation, add K^+, and centrifuge at once	79	40
15-min incubation, add K^+, and incubate 15 min more	51	44
15-min incubation, add K^+ + ATP, and incubate 15 min more	80	49

Effects of Different Cations and Anions—The experiment shown in Table IV was performed to determine whether ammonium ions, sulfate ions, or both were responsible for the increased loss of mitochondrial K^+. Ammonium chloride and ammonium acetate had no effect when P_i was omitted from the incubation medium. When P_i was present, both salts increased the loss of endogenous K^+, the loss being greater with ammonium acetate.

TABLE IV

Effects of various salts on loss of K^+ from rat liver mitochondria

Incubations were performed for 15 min at 37° in the standard incubation medium, which contained 10 mM P_i (when added) and 20.2 mg of mitochondrial protein. The salts were dissolved in 50 mM Tris-HCl, pH 7.4, before addition to the incubation medium. Other conditions were the same as in Table I.

Additions	Mitochondrial K^+ content	
	$-P_i$	$+P_i$
	mμmoles/mg protein	
None	42	52
10 mM ammonium sulfate	16	7
20 mM ammonium chloride	40	33
20 mM ammonium acetate	42	14
10 mM sodium sulfate	63	8
20 mM sodium chloride	40	46

TABLE V

Effects of sodium sulfate on loss of K^+ from rat liver mitochondria

In both experiments, incubations were performed for 15 min at 37° in the standard incubation medium, which contained 10 mM P_i (when added) and 19.1 and 18.9 mg of mitochondrial protein in Experiments 1 and 2, respectively. The salts were dissolved in 50 mM Tris-HCl, pH 7.4, before addition to the incubation medium.

Conditions	Mitochondrial K^+ content	
	$-P_i$	$+P_i$
	mμmoles/mg protein	
Experiment 1		
No incubation, no additions	84	82
No additions	39	50
1 mM sodium sulfate	49	48
2.5 mM sodium sulfate	55	41
5 mM sodium sulfate	61	24
10 mM sodium sulfate	61	7
Experiment 2		
No incubation, 60 mM sucrose	82	82
60 mM sucrose	37	42
30 mM sucrose + 10 mM ammonium sulfate	19	9
30 mM sucrose + 10 mM sodium sulfate	57	11
10 mM ammonium sulfate + 10 mM sodium sulfate	12	6
45 mM sucrose + 5 mM ammonium sulfate	28	12
45 mM sucrose + 5 mM sodium sulfate	52	35
30 mM sucrose + 5 mM ammonium sulfate + 5 mM sodium sulfate	13	8

The effects of sodium sulfate were strikingly different, depending on whether or not P_i was added to the medium. Without P_i, addition of sodium sulfate inhibited the loss of K^+. In the presence of P_i, addition of the salt increased the loss of K^+ to the same extent as did ammonium sulfate. Sodium chloride had no effect on mitochondrial K^+ loss whether or not P_i was added to the medium.

Effects of Sodium Sulfate—The data in Table IV suggested that the effects of ammonium sulfate could not be attributed to either the ammonium ion or the sulfate ion alone. Of particular interest was the action of sodium sulfate, the effects of which were studied in more detail. In an experiment similar to that presented in Fig. 1, the mitochondria were incubated in the standard incubation medium to which 10 mM P_i and 10 mM sodium sulfate were added. It was found that the curve for the loss of K^+ was strikingly similar to that observed with the same concentration of ammonium sulfate.

The first experiment in Table V shows that when P_i was omitted from the medium, addition of as little as 1 mM sodium sulfate resulted in an inhibition of K^+ loss, the maximum effect being observed between 5 and 10 mM.

Since the effects of sodium sulfate and ammonium sulfate were similar in a medium containing P_i but were strikingly different when P_i was omitted, the effects of a combination of both salts were studied. The results, which are shown in the second experiment in Table V, indicate that in the absence of P_i the effects of ammonium sulfate completely overshadowed those of sodium sulfate. Although the effects were not as striking in the presence of P_i, the combination of 5 mM sodium sulfate plus 5 mM ammonium sulfate also showed that the action of the ammonium salt completely masked that of the sodium salt.

Effects of Mg^{++}, $SO_4^{=}$, and P_i—In attempting to study the effects of pH on the action of sulfate ions, Mg^{++} had to be omitted since its presence at pH 8.4 caused the formation of a precipitate in the incubation medium. This led to the discovery that Mg^{++} markedly altered the effects of both salts on the loss of endogenous K^+ from liver mitochondria. Thus, when K^+ loss was studied at pH 7.4 without Mg^{++} in the standard incubation medium, both ammonium sulfate and sodium sulfate inhibited the loss of endogenous K^+ whether or not P_i was present in the medium.

A series of experiments was performed to study the effects of Mg^{++} at different concentrations of sulfate ions but in the absence of ammonium and sodium ions. Incubating media with different concentrations of sulfate ions were prepared by mixing 50 mM solutions of Tris-sulfate with 50 mM Tris-HCl at pH 7.4. The results, which are presented in Fig. 2, show that in the absence of P_i the loss of endogenous K^+ was progressively inhibited with increasing concentrations of sulfate. However, in the presence of P_i and at Mg^{++} concentrations above 2.5 mM, the loss of K^+ progressively increased as the concentration of sulfate was increased.

Since P_i had such a strikingly different effect on K^+ loss, depending upon whether sodium sulfate or ammonium sulfate was added to the incubation medium (*e.g.* Table V), experiments were performed to study the effects of increasing concentrations of P_i. Fig. 3 shows that when sulfate was added to the incubation medium without ammonium or sodium ions, the same concentration of P_i caused a progressively greater loss of mitochondrial K^+ at high concentrations of sulfate than at low sulfate concentrations.

Leakage of A_{259} Compounds—In considering various mechanisms of sulfate action, a change of mitochondrial membrane permeability appeared to be a possible explanation and one to which experimental evidence might be obtained. Experiments similar to those illustrated in Fig. 1 were therefore per-

formed in order to determine the effects of ammonium sulfate on the leakage from mitochondria of compounds showing an absorption maximum at 259 mμ (A_{259}), most of which are probably adenine nucleotides. The results, which are presented in Fig. 4, show that addition of ammonium sulfate to the incubation medium increased the release of A_{259} compounds whether or not P_i also was added to the medium. In another experiment, which is presented in Table VI, increased leakage of A_{259} compounds also occurred from mitochondria incubated for 15 min in the presence of 8 mM sulfate but without ammonium ions. This experiment also showed that the greatest loss of A_{259} compounds occurred in the sulfate medium when both P_i and Mg^{++} were added to the medium. In this connection it will be recalled that maximum loss of endogenous K^+ occurred with 8 mM sulfate when the incubation medium contained 10 mM P_i + 10 mM Mg^{++} (Figs. 2 and 3).

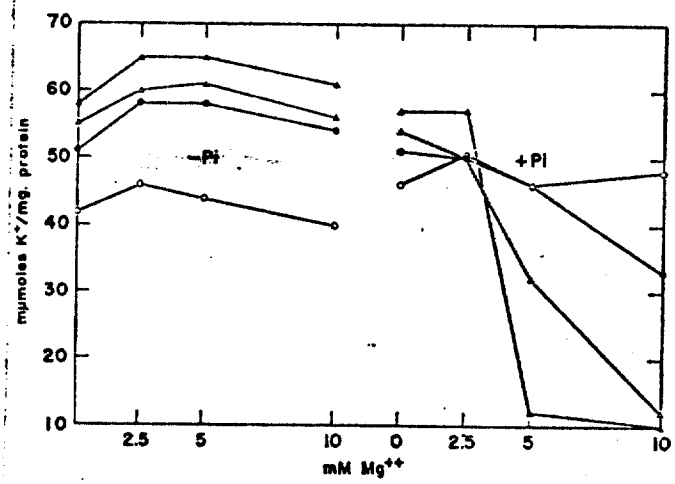

FIG. 2. The effects of Mg^{++} and $SO_4^{=}$ on the K^+ content of rat liver mitochondria. Mitochondria were incubated for 15 min at 37° in the standard incubation medium containing either 18.8 mg of mitochondrial protein ($-P_i$) or 20.4 mg of mitochondrial protein ($+P_i$). Different concentrations of sulfate were obtained by mixing 50 mM Tris-HCl, pH 7.4, with 50 mM Tris-sulfate, pH 7.4. The concentration of P_i, when added, was 10 mM. ○, no sulfate added; ●, 2 mM sulfate; △, 4 mM sulfate; ▲, 8 mM sulfate.

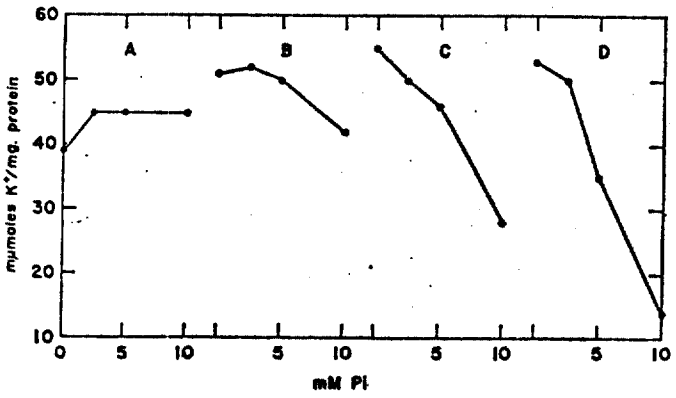

FIG. 3. The effects of P_i and sulfate on the K^+ content of rat liver mitochondria incubated for 15 min at 37° in the standard incubation medium to which had been added 20.2 mg of mitochondrial protein together with different concentrations of P_i. Different sulfate concentrations were obtained by mixing 50 mM Tris-HCl, pH 7.4, with 50 mM Tris sulfate, pH 7.4. *A*, no sulfate; *B*, 2 mM sulfate; *C*, 4 mM sulfate; *D*, 8 mM sulfate.

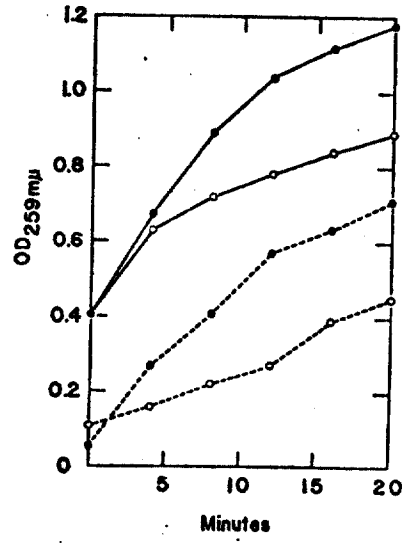

FIG. 4. The effects of ammonium sulfate on the leakage from rat liver mitochondria of compounds having an absorption maximum at 259 mμ. Mitochondria were incubated at 37° in the standard incubation medium, which also contained 10 mM ammonium sulfate, 10 mM P_i, and 19.6 mg of mitochondrial protein (●——●), or 10 mM ammonium sulfate, no P_i, and 17.6 mg of mitochondrial protein (●- - -●). ○——○ and ○- - -○, 30 mM sucrose was added to the medium in addition to the sucrose already present. For further details, see "Experimental Procedure."

TABLE VI

Effects of sulfate ions on leakage of A_{259} compounds from rat liver mitochondria

Mitochondria (21.5 mg of protein) were incubated for 15 min at 37° and pH 7.4 in the standard incubation medium. A concentration of 8 mM sulfate was obtained by mixing 50 mM Tris-HCl, pH 7.4, with 50 mM Tris-sulfate, pH 7.4. For further details, see the text.

Conditions	Absorbance at 259 mμ	
	Control	Sulfate
Complete system	0.56	0.84
-P_i	0.41	0.61
-Mg^{++}	0.51	0.58
-P_i, -Mg^{++}	0.46	0.57

DISCUSSION

A possible explanation for the increased loss of K^+ induced by sulfate ions is a permeability change in the mitochondrial membrane. If this did occur, then the results of the present study suggest that sulfate increases mitochondrial membrane permeability to K^+ leaking out of the mitochondria.

An increased permeability to K^+ and to other substances leaking out of the mitochondria could explain the delay in onset of the increased K^+ loss when mitochondria were added to a sulfate-containing medium (*cf.* Fig. 1). Thus it was shown that ammonium sulfate or sulfate ions alone increased the loss of A_{259} compounds from the mitochondria. If one assumes that membrane permeability was increased as soon as the mitochondria came into contact with sulfate ions, then endogenous substrates and nucleotides probably began leaking out of the mitochondria at an increased rate. When the ccu-

centration of these compounds reached a level low enough so that sufficient metabolic energy could not be generated endogenously to retard the loss of K^+, the metal ion would begin to leak out of the mitochondria at a greatly increased rate.

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Die Resorption aus dem Darm von isotonischen Lösungen von Glucose und Sorbose, verglichen mit der von Natriumsulfat.

Von

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(Eingegangen am 2. Dezember 1934.)

Mit 5 Abbildungen im Text.

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Nachdem aus verschiedenen Untersuchungen mit Sicherheit hervorgegangen war, daß Glucose selektiv rasch aus dem Darm verschwindet, war es von Interesse zu untersuchen, inwiefern die Resorptionsgeschwindigkeit die Diffusionsgeschwindigkeit übersteigt. Wir vergleichen die Resorptionsgeschwindigkeit isotonischer Lösungen von Glucose mit der entsprechender Lösungen von Na_2SO_4. Durch eine Pergamentmembran (*Schleicher & Schüll* Nr. 579) diffundieren diese mit nahezu gleicher Geschwindigkeit. Im lebenden Darm ist es aber anders.

Von den Sulfaten liest man allgemein, daß sie besonders langsam resorbiert werden. Diese langsame Resorbierbarkeit soll auch die Erklärung dafür sein, daß sie als Abführmittel wirken. Von *Höber*[1] wird angegeben, daß aus isotonischen Lösungen Traubenzucker aus dem Darm rascher resorbiert wird als Magnesiumsulfat. Dabei hat Magnesiumsulfat ein etwas geringeres Molekulargewicht (Glucose 180, $MgSO_4$ 120) und sollte deshalb eher rascher resorbiert werden.

Die langsame Resorption von Sulfaten wird verschieden erklärt. Einmal so, daß sie kolloidale Verbindungen bilden und dadurch die Membran verdichten; dann so, daß sie mit Calcium wasserunlösliche Verbindungen geben. Mit einer Membranverdichtung ist die langsame Resorption jedoch nicht zu erklären, wie schon die Versuche von *Knaffl-Lenz* und *Nogaki*[2] zeigten. *Wallace* und *Cushny*[3] erklärten die langsame Resorption von Sulfaten damit, daß diese mit Calcium wenig wasserlösliche Verbindungen bilden, die schlecht diffusibel sind.

Es scheint, daß eine beständige Quelle der Mißverständnisse bei der Erforschung dieser Frage die war, daß Glucose zum Vergleich herangezogen wurde, gerade jener Zucker, für dessen Resorption aus dem Darm ganz besondere Verhältnisse vorliegen. Hat man mit diesem verglichen, so fand man, daß isotonische Lösungen anderer Substanzen langsamer resorbiert werden. Dies beruht aber gar nicht auf der Eigen-

[1] *R. Höber*, Pflügers Arch. 74, 246, 1899. — [2] *E. Knaffl-Lenz* u. *S. Nogaki*, Arch. f. exper. Pathol. 105, 109, 1925. — [3] *Wallace* u. *Cushny*, Pflügers Arch. 77, 702, 1899.

Sulf.

schaft der Salze, sondern auf einer spezifisch raschen Resorption des Vergleichsobjektes, der Glucose, woran schon *Höber* (l. c.) gedacht hat.

Zum weiteren Vergleich wird deshalb in einem späteren Teil eine andere Hexose (Sorbose) untersucht, für welche keine selektiv rasche Resorption wie für Glucose besteht.

Versuche.

Das Molekulargewicht der Glucose (180) ist etwas höher als das von Na_2SO_4 (142). Zum Vergleich werden aber natürlich nicht äquimolare, sondern isotonische Lösungen benutzt. Eine solche ist eine 5,5%ige Glucose- und eine 1,9%ige Na_2SO_4-Lösung. Beide sind ferner isotonisch mit dem Blutserum der Säuger bzw. einer physiologischen 0,85%igen NaCl-Lösung[1].

Im Anhang ist die Diffusion von Glucose und Na_2SO_4 durch nicht lebende Membranen geprüft. Wir nehmen hier das Resultat dieser Versuche vorweg: aus isotonischen Lösungen diffundiert bei Anwendung der verschiedensten nichtlebenden Membranen das Na_2SO_4 rascher als Glucose.

Ganz anders liegen die Verhältnisse jedoch im lebenden Darm, wie sich aus den folgenden Versuchen ergibt.

Erste Versuchsreihe.

Zuerst wurde geprüft, wie sich im lebenden Tier die Resorption aus dem Darm verhält, wenn man in eine abgebundene Darmschlinge isotonische Lösungen von Glucose und Natriumsulfat bringt.

Wir sind zuerst so vorgegangen, daß wir bei Kaninchen den Darm am Pylorus und am Dickdarm unterbanden. Die Länge des abgebundenen Darms war 140 bis 160 cm. Die Tiere hatten ein Gewicht von 2660 bis 2750 g. Sie hungerten vor dem Versuch 48 Stunden. Zum Versuch erhielten sie 0,7 ccm Numal intravenös. In den Darm wurde mittels einer Pravazspritze ein Gemisch bestehend aus 10 ccm isotonische Glucoselösung (550 mg Glucose in destilliertem Wasser) und 10 ccm isotonische Natriumsulfatlösung (190 mg Na_2SO_4 in destilliertem Wasser) injiziert. Beide Lösungen und also auch ihr Gemisch sind isotonisch. In jedes Tier wurde nur eine Injektion gemacht und das Tier nach einer bestimmten Zeit, welche zwischen 30 und 240 Minuten lag, durch intrakardiale Chloroforminjektion getötet. Dann wurde der Darm vom Mesenterium abgeschnitten, mit destilliertem Wasser wiederholt ausgewaschen, der Inhalt filtriert und analysiert.

Zur Bestimmung des Zuckers wurde die Methode von *Bertrand* benutzt und immer Doppelanalysen ausgeführt, welche gute Übereinstimmung zeigten. Zur Sulfatbestimmung haben wir in früheren Versuchen das Sulfat ausgefällt und im Platintiegel geglüht, in späteren Versuchen wurde es nach der Fällung durch einen *Gooch*-Tiegel filtriert und darin gewogen.

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[1] *S. Landolt-Börnstein*, Tabelle 2, S. 1129.

Die Versuchsresultate sind in der Tabelle I zusammengestellt (Versuche 7 bis 23).

Tabelle I.

Versuchs-Reihe	Versuchs-Nr.	Dauer des Versuchs Min.	Länge des benutzten Darmstückes cm	Glucose injiziert mg	Glucose wiedergefunden mg	Glucose resorbiert mg	Na_2SO_4 injiziert mg	Na_2SO_4 wiedergefunden mg	Na_2SO_4 resorbiert mg	In der Schleimhaut gefundenes Na_2SO_4 mg
1	7	30	ganzer Darm	280	104	176	90	58,5	31,5	
	8	60	163	552	102	450	190	91,2	97,8	
	9	90	ganzer Darm	551	41	510	189	71	118	
	10	120	" "	550	0	550	191	53	138	
	11	120	140	551	125	426	190	51	127	12
	12	150	170	550	26,7	521,3	189	37	138	14
	13	160	160	550	17,7	532,3	189	21	168	
	14	180	150	550	0	550	190	23	156	11
	15	240	150	551	0	551	189	21,5	167,5	
	20	60	140	550	117	433	190	91,7	98,3	
	21	90	150	550	105	445	190	75,8	114,2	
	23	120	160	550	50	500	190	54	136,0	
2	24	60	70	550	340	210	190	147,5	42,5	
	25	60	70	550	348	202	190	148,0	41,1	
	26	90	70	550	262,7	287,3	190	134,4	55,6	
	27	90	70	550	239,9	310,1	190	130,3	59,7	
	28	120	70	550	159,9	390,1	190	121,9	68,1	
	29	120	70	550	118,3	431,7	190	117,8	72,2	
	30	150	70	550	99,1	450,9	190	108,7	81,3	
3	38	60	70	550	345,0	205	—	—	—	
	39	60	70	550	338,0	212	—	—	—	
	40	60	70	—	—	—	190	145,0	45,0	
	41	60	70	—	—	—	190	141,7	48,3	

Zweite Versuchsreihe.

Außer diesen zwölf Versuchen, bei welchen die ganzen Dünndärme benutzt worden sind, wurde noch eine zweite Serie von Versuchen ausgeführt. Zu diesen Versuchen sind weitere sieben Tiere benutzt worden (Versuche 24 bis 30). Die Methodik war dieselbe wie in der vorigen Versuchsreihe, jedoch mit dem Unterschied, daß immer genau gleich lange Dünndarmstücke gewählt wurden, was sehr wichtig erscheint, wenn man unter gleichen Verhältnissen miteinander vergleichbare Resultate erhalten will. Hierzu wurde 2 cm unterhalb des Pylorus der Dünndarm unterbunden und ein genau 70 cm langes Stück abgemessen und dort zum zweitenmal unterbunden.

Sowohl aus der ersten wie aus der zweiten Versuchsreihe geht hervor, daß aus der isotonischen Lösung von Glucose viel mehr resorbiert wird als von den entsprechenden Natriumsulfatlösungen. Dieses Resultat ist außer in den Tabellen in Abb. 1 und 2 dargestellt, wo der zeitliche Ablauf der Resorption aus den einzelnen Versuchen zusammengestellt ist.

Man kann die Unterschiede in der Resorptionsgeschwindigkeit noch deutlicher machen, wenn man die Zeiten vergleicht, nach welchen die Hälfte der eingegebenen Substanz resorbiert ist. Diese „Halbzeiten" sind in den Abbildungen durch einen Strich angegeben. Sie zeigen, daß der Traubenzucker rascher resorbiert wird als das Sulfat, das länger liegen bleibt. Andererseits besteht aber gar keine Ursache zu behaupten, das Sulfat werde nicht resorbiert. Im Laufe von 6 Stunden sind die eingegebenen Mengen fast ganz aus dem Darm verschwunden, wie aus Abb. 1 hervorgeht.

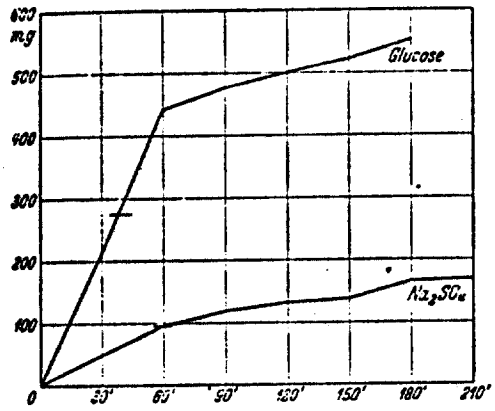

Abb. 1.
Resorption aus ganzem Kaninchendarm von blutisotonischen Glucose- und Na_2SO_4-Lösungen.

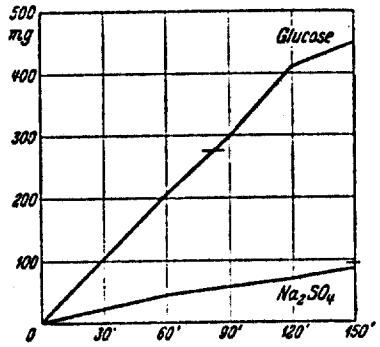

Abb. 2.
Resorption aus abgebundenem Kaninchendarm von blutisotonischen Glucose- und Na_2SO_4-Lösungen.

Dritte Versuchsreihe.

Es ergab sich nun die Frage, ob im lebenden Tier durch die Zugabe des Sulfats nicht auch die Resorptionsgeschwindigkeit für Glucose vermindert wird, bzw. ob umgekehrt nicht der Zucker die Resorptionsgeschwindigkeit für das Natriumsulfat beeinflußt. Für diese Versuche sind wir deshalb so vorgegangen, daß, nach der in der zweiten Versuchsreihe benutzten Methodik, in den Versuchen 38 und 39 ein genau 70 cm langes Darmstück genommen wurde und in dieses 10 ccm einer isotonischen Glucoselösung mit einem Gehalt von 550 mg in destilliertem Wasser, und in Versuchen 40 und 41 10 ccm isotonische Sulfatlösung enthaltend 190 mg Na_2SO_4 gegeben wurde.

Die Tabelle I enthält die diesbezüglichen vier Versuche (Versuche 38 bis 41), welche klar zeigen, daß bei 60 Minuten Versuchsdauer die resorbierten Mengen genau dieselben sind, sowohl für Glucose allein, als auch für das Na_2SO_4 allein, wie wenn die beiden zusammen eingegeben werden. Zum Vergleich dienen Versuche 24 und 25 der vorigen Versuchsreihe, zu welchen diese Versuche genaue Parallelversuche bilden. Das Resultat dieser Versuchsreihen ist also, daß aus einem

Gemisch von Na_2SO_4 und Glucose beide Substanzen mit derselben Geschwindigkeit resorbiert werden, als wenn sie allein vorhanden sind. Es sei hier schon bemerkt, daß dieses Resultat gegen die Annahme spricht, daß durch das Sulfat eventuell eine verdichtende Wirkung auf die resorbierende Membran zustande kommt, denn dann müßte auch der Zucker langsamer resorbiert werden als ohne Sulfat.

Vierte Versuchsreihe.

Wir hatten also gefunden, daß im lebenden Tier aus isotonischen Lösungen von Glucose und Na_2SO_4 letzteres langsamer resorbiert wird. Es sollte nun untersucht werden, ob der Nachweis dieser Resorptionsverlangsamung auch in überlebenden Därmen gelingt.

Zu diesen Versuchen sind wir nach der Methode von *Macleod* und *Magee*[1] vorgegangen. Das Kaninchen hungerte vor dem Versuch 38 bis 48 Stunden. Dann wurde es durch Köpfschlag getötet. Der Dünndarm wurde sogleich vom Mesenterium abgelöst und in 38° C Tyrodelösung gelegt. Dann wurde er in 15 cm lange Stücke zerschnitten; das untere Ende dieser Stücke wurde doppelt unterbunden; in das obere Ende wurde eine Glaskanüle eingebunden. Nun kam der Darm in ein Glasgefäß mit einem Inhalt von 70 ccm Tyrodelösung ohne Glucose. Das Innere des Darms wird durch die Glaskanüle mit 4 oder 5 ccm Lösung gefüllt. In einer Versuchsreihe wurde diese Lösung hergestellt aus gleichen Mengen einer isotonischen Glucoselösung und einer Tyrodelösung ohne Glucose. In anderen Versuchen kam in das Innere des Darms eine Lösung, die zur Hälfte aus einer isotonischen Glucoselösung und zur Hälfte aus einer isotonischen Na_2SO_4-Lösung bestand.

In der Tabelle II sind die absoluten Mengen von Glucose und Sulfat angegeben, welche in das Innere des Darms gebracht wurden. Die Därme befanden sich während der ganzen Zeit in sauerstoffdurchperlter Tyrodelösung ohne Zucker in *Magnus*schen Gefäßen. Die Versuchsdauer betrug 30 bis 90 Minuten. Nach den Befunden von *Magee* usw. kann man nur bei einer Versuchsdauer von 30 Minuten die Darmschleimhaut für lebend betrachten. Es ist ferner bei diesen Versuchen immer wichtig, wie weit das Darmstück vom Pylorus entfernt ist, denn verschieden weit entfernte Darmstücke sind bezüglich ihrer Resorptionsgeschwindigkeit nicht gleichwertig. Besonders beim Benutzen von weit voneinander entfernten Darmstücken muß man beim Vergleich der Resultate hierauf sehr achten.

In vielen Versuchen wurde am Ende des Versuchs die Glucose- und Sulfatmenge, sowohl innen wie außen bestimmt. In anderen Versuchen begnügten wir uns nur mit einer Bestimmung in der Außenflüssigkeit. In einzelnen Versuchen wurde am Ende noch die Schleimhaut abgestrichen und auch hierin eine Sulfatanalyse gemacht.

Die Bestimmung des Zuckers geschah nach der Methode von *Bertrand*. Die Sulfatbestimmungen wurden in Versuchen 42 bis 57 durch Verbrennung im Platintiegel und in den Versuchen 58 bis 63 durch Wägung im *Gooch*-Tiegel ausgeführt, so wie oben beschrieben.

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[1] *Macleod, Auchinachie* u. *Magee*, J. of Physiol. 68, 67, 1929.

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Methode von *Bertrand*.
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urch Wägung im *Gooch-*

ol. 68, 67, 1929.

Tabelle II.

Versuch	Tier	Untersucht nach Tötung des Tieres	Dauer des Versuchs	Entfernung des Darmstücks vom Pylorus	Glucose: injiziert	Glucose: innen wiedergefunden	Glucose: außen wiedergefunden (diffundiert)		Na_2SO_4: injiziert	Na_2SO_4: innen wiedergefunden	Na_2SO_4: außen wiedergefunden (diffundiert)		Na_2SO_4: in der Schleimhaut wiedergefunden
Nr.	Nr.	Min.	Min.	cm	mg	mg	mg	%	mg	mg	mg	%	mg
42	I	5	30	5	137,5	112,5	9,8	7,1	47,5	36,6	5,0	10,7	3,3
43	I	5	30	20	137,5	112,0	9,2	6,6	47,5	36,0	5,3	11,1	3,0
44	II	5	30	5	137,5	109,0	9,6	6,9	47,5	38,2	5,0	10,7	—
45	II	5	30	20	137,5	105,3	10,6	7,6	47,5	35,0	5,7	12,0	3,0
46	III	5	30	5	137,5	—	9,0	6,4	47,5	—	4,8	10,1	2,8
47	III	5	30	20	137,5	—	9,5	6,7	47,5	—	5,5	11,5	—
58	XI	5	30	5	137,5	115,2	8,2	5,9	47,5	37,3	4,2	8,9	—
59	XI	5	30	20	137,5	112,7	8,4	6,1	47,5	35,7	4,5	9,5	—
60	XI	5	30	35	137,5	120,3	7,5	5,5	47,5	36,9	4,2	8,9	—
61	XI	5	30	50	137,5	115,1	9,0	6,6	47,5	36,8	4,0	8,4	—
64	IV	5	30	5	137,5	—	11,0	8,0	47,5	—	—	—	
68	VII	5	30	5	137,5	—	13,5	9,8	47,5	—	—	—	
69	VII	35	30	20	137,5	—	12,8	9,3	47,5	—	—	—	
75	VIII	5	30	—	162,0	—	10,0	6,2	57,0	—	4,4	7,0	
							Mittel:	7,0				9,9	
48	III	35	60	35	110	80	14,8	13,4	38,0	26,5	6,7	17,0	2,4
49	III	35	60	50	110	78,3	15,6	14,0	38,0	26,0	7,0	18,4	—
50	V	5	60	20	110	—	11,2	10,0	38,0	—	7,1	18,5	—
62	XIII	5	60	5	137,5	—	13,7	9,6	47,5	—	6,5	13,7	—
63	XII	5	60	20	137,5	105,3	13,0	9,5	47,5	35,3	7,0	14,8	—
65	IV	30	60	20	137,5	—	25,0	18,2	47,5	—	—		
							Mittel:	12,4				16,5	
52	IV	40	90	160	110,0	—	19,0	17,2	38,0	—	—	—	—
53	IV	60	90	175	110,0	—	20,0	18,1	38,0	—	10,3	27,1	—
54	V	5	90	5	110,0	—	16,4	14,9	38,0	—	10,6	27,5	—
							Mittel:	16,7				27,3	

Die Resultate sind in der Tabelle II zusammengestellt. Ferner ist in der Abb. 3 die Diffusion von Glucose und Na_2SO_4 aus dem isolierten Kaninchendarm nach den Mittelwerten auch graphisch dargestellt.

Es zeigt sich, daß regelmäßig das Sulfat dem Zucker voraneilt, also gerade umgekehrt wie im lebenden Tier. Das geht schon aus den Prozentwerten hervor, die zeigen, daß in jedem Zeitmoment prozentual mehr Sulfat als Dextrose in der Außenflüssigkeit vorhanden ist. Freilich können diese Diffusionsversuche nicht solange durchgeführt werden, wie am lebenden Tier, weil, wie erwähnt, die Därme bald absterben.

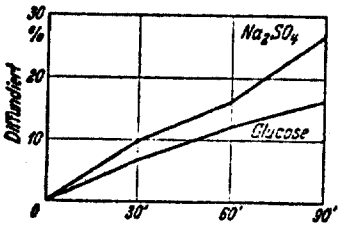

Abb. 3.
Diffusion von isotonischen Glucose- und Na_2SO_4-Lösungen durch überlebenden Kaninchendarm in Prozent der Menge.

Dieses Resultat, daß aus isotonischen Lösungen das Sulfat nicht nur nicht langsamer, sondern rascher diffundiert, steht also im Gegensatz zu den Versuchen am lebenden Tier. Es zeigt, daß wir es hier nicht mehr mit den natürlichen Verhältnissen zu tun haben, sondern mit solchen, die der rein physikalischen Diffusion durch nicht lebende Membranen entsprechen.

Fünfte Versuchsreihe.

Resorptionsversuche am isolierten überlebenden Darm ohne Sulfatzusatz. Diese Versuchsreihe ist als Parallele zu der Versuchsreihe 4 zu betrachten. Bei fünf Versuchen ist angegeben, zu welchen Versuchen sie als Parallelversuche (am selben Tier) dienten. Im allgemeinen geht aus diesen Versuchen hervor, daß die Diffusionsgeschwindigkeiten in diesen Därmen für Glucose nicht gleich sind mit jenen der vierten Versuchsreihe, wenn Sulfat hinzugesetzt ist. Unter dem Einfluß des Sulfats läßt sich eine Verlangsamung der Zuckerresorption nachweisen. Das steht im Gegensatz zu dem, was wir in Versuchsreihe 1 und 2 bezüglich der Resorption des Zuckers am ganzen Tier gefunden haben. Dort war, wie erwähnt, und wie speziell aus der Versuchsreihe 3 hervorgeht, die Resorption des Zuckers innerhalb 60 Minuten nicht schlechter bei Zusatz von Sulfat als ohne dieses. Das zeigt ein Vergleich der Versuche 24 und 25 (mit Sulfat) mit Versuchen 38 und 39 (205, 212 mg).

Die Versuche am sogenannten „überlebenden Darm" haben also wesentliche Unterschiede gegenüber dem normalen Darm gezeigt. Die langsamere Diffusion des Sulfats gegenüber der Glucose fehlt hier, und ferner zeigt sich, daß die Glucose unter dem Einfluß des Sulfats langsamer diffundiert. Es ist merkwürdig, daß nach den Versuchen von *Macleod, Magee* und *Auchinachie* (l. c.) der überlebende Darm beim Vergleich von Dextrose und Xylose sich noch wie ein lebender Darm

Tabelle III.

Versuch Nr.	Tier Nr.	Untersucht nach Tötung des Tieres Min.	Dauer des Versuchs Min.	Dextrose: Injektion mg	Dextrose: außen wiedergefunden (diffundiert) mg	Dextrose: außen wiedergefunden (diffundiert) %	Parallele zu Versuch Nr.	Bemerkungen
55	VI	5	30	137,5	14,1	10,5	—	Halbisotonisch Tyrode und Glucose 5 ccm
56	VI	5	30	137,5	15,5	11,3	—	Dasselbe
57	VI	35	30	137,5	15,0	10,9	—	„
79	VIII	5	30	162,0	17,0	10,5	75	Halbisotonisch Tyrode und Glucose 6 ccm
81	IV	5	30	137,5	23,0	16,7	64	Halbisotonisch Tyrode und Glucose 5 ccm
82	IV	30	60	137,5	31,0	22,6	65	Dasselbe
85	VII	5	30	137,5	18,8	13,7	68	„
86	VII	35	30	137,5	17,0	12,4	69	„
				Mittel:		12,3		

benommen hat. Bezüglich Na_2SO_4 ist jedoch in unseren Versuchen das nicht mehr der Fall gewesen.

Sechste Versuchsreihe.

Hemmung der Dextroseresorption durch Monojodessigsäure. Aus Untersuchungen von *Wilbrandt* und *Laszt*[1] war hervorgegangen, daß die Bevorzugung der Dextrose bei der Resorption gegenüber anderen Zuckern in spezifischer Weise durch Monojodessigsäure gehemmt werden kann. Ohne daß wir hier darauf eingehen, was die Ursache dieser Bevorzugung der Dextrose ist, sei hier nur darauf hingewiesen, daß es wahrscheinlich erscheint, daß wir hier eine synthetische Funktion des Epithels hemmen. Es lag deshalb der Gedanke nahe, daß auch die Bevorzugung der Dextrose gegenüber dem Sulfat durch diesen vitalen Prozeß des Epithels bedingt sei. Deshalb wurde untersucht, ob es möglich ist, die Differenz in der Resorptionsgeschwindigkeit zwischen einer blutisotonischen Glucoselösung und einer ebenfalls blutisotonischen Na_2SO_4-Lösung dadurch zum Verschwinden zu bringen, daß das Tier mit Monojodessigsäure vergiftet wurde.

Es wurden zu diesen Versuchen Ratten benutzt, und zwar wurde bei diesen ein 60 cm langes Dünndarmstück abgemessen und mit Tyrodelösung ausgewaschen. Die Tiere waren mit 12 mg Urethan (1 ccm 25 % subcutan) narkotisiert. Der Darm wurde dann mit 6 ccm Flüssigkeit, enthaltend 165 mg Dextrose und 57 mg Na_2SO_4 gefüllt. Nach 1 Stunde wurde das Tier getötet und der Darm ausgewaschen, und die Flüssigkeit auf ihren Gehalt an Dextrose und Sulfat analysiert. Die Tabelle IV stellt bei drei Tieren mit Vergiftung mit Monojodessigsäure und bei drei Versuchstieren ohne Monojodessigsäure die Prozentwerte der resorbierten Dextrose und

[1] *W. Wilbrandt* u. *L. Laszt*, diese Zeitschr. 259, 398, 1933.

Na_2SO_4 zusammen. Die Monojodessigsäure wurde 2 Stunden vor dem Resorptionsversuch in 10%iger Lösung subcutan in Dosen von 0,12 bis 0,16 mg pro g Tier gegeben.

Tabelle IV.

Versuch Nr.	Gewicht des Tieres g	Ohne Vergiftung	Resorbiert Dextrose %	Resorbiert Na_2SO_4 %	Versuch Nr.	Gewicht des Tieres g	Vergiftet mit Monojodessigsäure mg	Resorbiert Dextrose %	Resorbiert Na_2SO_4 %
101	210	—	92,7	13,5	102	190	0,14	18,0	14,1
103	145	—	90,0	12,6	104	150	0,14	18,3	13,5
105	140	—	75,7	15,2	106	145	0,16	22,1	14,9
		Mittel:	86,8	13,8			Mittel:	19,5	14,2

Es zeigt sich, daß bei normalen unvergifteten Ratten unter diesen Versuchsbedingungen in der gleichen Zeit etwa sechsmal soviel Glucose als Sulfat resorbiert wird. Wie aus der Tabelle IV ferner hervorgeht, wird durch eine Allgemeinvergiftung mit Monojodessigsäure eine sehr charakteristische Reaktion ausgelöst. Diese besteht darin, daß die Resorptionsgeschwindigkeit des Sulfats sich nicht ändert, was der Beweis dafür ist, daß keine Allgemeinschädigung des Tieres vorliegt, dagegen geht die Resorptionsgeschwindigkeit der Dextrose nun zurück auf annähernd denselben Wert wie der des Sulfats. Demnach beruht die Bevorzugung der Resorption der Dextrose auf gewissen Umbauprozessen im Darmepithel, welche durch Monojodessigsäure gehemmt werden. Nach dieser Versuchsreihe handelt es sich also bei der bekannten Erscheinung, daß aus isotonischen Lösungen Glucose rascher resorbiert wird als Sulfat, nicht um eine Verlangsamung spezifischer Art für die Resorption des Sulfats, sondern im Gegenteil um eine spezifische Beschleunigung für die Dextroseresorption, und unser Problem liegt dann nicht darin, die Verlangsamung der Sulfatresorption zu erklären, sondern mehr darin, eine Erklärung für die Beschleunigung der Dextroseresorption zu suchen.

Siebente Versuchsreihe.

Die Resorption von Sorbose. In den vorigen Versuchsreihen haben wir die Resorption von Dextrose mit einer isotonischen Na_2SO_4-Lösung verglichen. Dieser Vergleich könnte deshalb nicht einwandfrei sein, weil bezüglich des Sulfats besondere Verhältnisse vorliegen könnten. Deshalb mußte die Resorption von Dextrose mit einem anderen Zucker verglichen werden. Als solchen wählten wir l-Sorbose. Sie wird im Körper so wie die Pentosen und nicht so gut wie Dextrose verwendet, und ein Teil wird wieder ausgeschieden. Ihrer chemischen Struktur nach steht sie der Fructose nahe.

Nachdem in Versuchen von *Wilbrandt* und *Laszt* (l. c.) die Resorption von verschiedenen Hexosen und Pentosen bereits verglichen wurde,

führten wir mit genau derselben Methodik die folgenden Versuche aus, welche also mit den Ergebnissen dieser direkt vergleichbar sind. Die Methodik ist oben ausführlich besprochen.

In allen Versuchen wurde eine blutisotonische Sorboselösung benutzt. Diese enthält 5500 mg Sorbose in 100 ccm, wobei angenommen wird, daß diese Lösung isotonisch ist mit einer äquimolaren Dextroselösung. Von dieser wurden Ratten von etwa 200 g Körpergewicht 3 ccm in eine 30 cm lange Dünndarmschlinge injiziert und die Resorption während 1 Stunde beobachtet.

Wie aus der Tabelle V hervorgeht, werden von der Sorbose unter diesen Umständen 22 % resorbiert. Wir vergleichen dieses Resultat mit den Werten von verschiedenen Zuckern, wie sie nach genau derselben Methodik von *Wilbrandt* und *Laszt* (l. c.) gewonnen wurden. Nimmt man die Glucosemenge, die unter den gleichen Bedingungen resorbiert wird, zu 100, so wird von Sorbose 30 resorbiert, und stellt man die Resorptionsgeschwindigkeiten der verschiedenen Zucker in eine Reihe, so zeigt sich, daß die Sorbose ebenso rasch wie die Xylose, also auch dreimal langsamer als Dextrose, resorbiert wird.

Vergleicht man aber nun diesen Wert mit dem in der vorigen (sechsten) Versuchsreihe (Tabelle IV) gewonnenen, so ergibt sich ferner das wichtige Resultat, daß die Sorbose kaum besser resorbiert wird als das Na_2SO_4 aus einer isotonischen Lösung. Xylose, Sorbose und Na_2SO_4 verhalten sich also bezüglich ihrer Resorptionsgeschwindigkeiten nahezu gleich und andererseits ist diese drei- bis sechsmal geringer als die von Dextrose.

Diese Parallelität geht noch weiter, wenn man die Wirkung von Monojodessigsäure untersucht. In der vorigen (sechsten) Versuchsreihe ist gezeigt worden, daß Monojodessigsäurevergiftung die Resorptionsgeschwindigkeit des Na_2SO_4 nicht beeinflußt, während die Dextroseresorption ungefähr auf den Wert des Sulfats herabgedrückt wird. Von *Wilbrandt* und *Laszt* (l. c.) war dasselbe bezüglich der Resorption der Mannose, Xylose und Arabinose gezeigt worden, ebenfalls im Gegensatz zur Dextrose. In den nächsten vier Versuchen (Tabelle V) ist deshalb an vier Tieren, die ebenfalls wie in den früheren Versuchen untersucht wurden, die Resorption geprüft, wenn die Tiere vorher mit 0,12 mg pro g Monojodessigsäure subcutan vergiftet worden waren.

Wie die Tabelle V zeigt, hat sich bezüglich der Resorptionsgeschwindigkeit gar nichts geändert. Monojodessigsäure ist also auf die Resorption von Sorbose ebenso unwirksam wie auf die von Xylose oder Na_2SO_4. Verglichen mit einer Glucoseresorption von 100 finden wir jetzt für Sorbose 31,6. Der Quotient unvergiftet/vergiftet beträgt also 30/31,6 = 1. Das ist dasselbe Resultat wie mit Mannose, Xylose und Arabinose in den Versuchen von *Wilbrandt* und *Laszt*.

Wir kommen also zu dem Resultat, daß Sorbose, trotzdem sie eine Hexose ist, ebenso wie Xylose, aber auch ebenso wie das anorganische

Tabelle V. Resorption von l-Sorbose aus dem Darm.

Versuch Nr.	Resorbiert aus der 165 mg injizierten Sorbosemenge in einer Stunde			
	ohne Jodessigsäure		mit Jodessigsäure	
	mg	%	mg	%
1—8	35,3	21,4	41,7	25,3
	31,7	19,8	41,7	25,3
	41,7	25,3	34,6	21,0
	35,3	21,4	35,3	21,4
Mittel:	36,0	22,0	38,3	26,2

Na_2SO_4 einfach ihrer Diffusionsgeschwindigkeit entsprechend resorbiert wird. Umgekehrt ergibt sich dann, daß für die besondere selektive Beschleunigung der Dextrose ein aktiver Prozeß, ein eventueller Umbau in der Darmschleimhaut angenommen werden muß. Für die Analyse unserer Versuche mit Na_2SO_4 ergibt sich das Resultat, daß dieses Salz durchaus nicht „spezifisch langsam" resorbiert wurde, sondern ganz seiner Diffusionsgeschwindigkeit entsprechend, etwa so wie eine isotonische Lösung von Sorbose.

Zusammenfassung.

1. Im lebenden Tier wird aus einem blutisotonischen Dextrose- und Na-Sulfatgemisch, in welchem beide Substanzen in isotonischen Mengen vorhanden sind, die Dextrose rascher resorbiert als das Sulfat, was sowohl bei Benutzung des ganzen Darms, als auch bei Benutzung von gleich langen Dünndarmschlingen gefunden wurde.

2. Im lebenden Tier beeinflußt Na_2SO_4 die Resorptionsgeschwindigkeit der Dextrose nicht und die Dextrose beeinflußt auch die Resorptionsgeschwindigkeit des Sulfats nicht.

3. Die Dextrose wird etwa fünfmal so rasch wie das Na_2SO_4 aus isotonischer Lösung resorbiert. In 1 Stunde z. B. 450 mg Dextrose gegenüber 90 mg Na_2SO_4 (Abb. 1) oder 210 mg Dextrose gegenüber 45 mg Na_2SO_4 (Abb. 2).

4. Aus dem 1 Stunde „überlebenden" Darm diffundiert umgekehrt das Sulfat rascher als die Dextrose. Die Därme benehmen sich also wie nicht lebende Membranen.

5. In 1 Stunde „überlebenden" Därmen hemmt Sulfat die Diffusion gleichzeitig vorhandener Dextrose.

6. Die Resorptionsbeschleunigung für Dextrose gegenüber Sulfat wird durch Monojodessigsäure gehemmt. Die Resorptionsgeschwindigkeit des Na_2SO_4 wird dabei nicht beeinflußt.

7. Sorbose wird bedeutend langsamer resorbiert als Glucose, etwa ebenso langsam wie Xylose oder isotonische Na_2SO_4-Lösung. Ihre Resorptionsgeschwindigkeit wird durch Monojodessigsäure nicht gehemmt.

8. Die Monojodessigsäure hemmt danach einen Prozeß in der lebenden Darmschleimhaut, der die Dextroseresorption begünstigt. Die langsame Resorption von Na_2SO_4 entspricht der physikalischen Diffusion dieser Substanz.

Anhang.

Umkehr des Diffusionsverhältnisses einer isotonischen Glucose- und Na_2SO_4-Lösung durch tote Doppelmembranen.

Eine isotonische Lösung von 5,5 % Glucose bzw. 1,9 % Na_2SO_4 wird in eine Dialysierhülse von *Schleicher & Schüll* Nr. 579 (Pergament) gebracht. Die Diffusion gegen dest. Wasser verläuft wie Abb. 4 es zeigt. Die Glucose diffundiert langsamer als das Na_2SO_4. Die halbe Menge („Halbwertzeit") ist für letztere bedeutend früher erreicht als für erstere. Na_2SO_4 diffundiert immer 20 bis 30 % rascher. Andere Membranen (Schweinsblasen, Kalb-, Schwein-, Schafcoecum und Kollodium usw.)

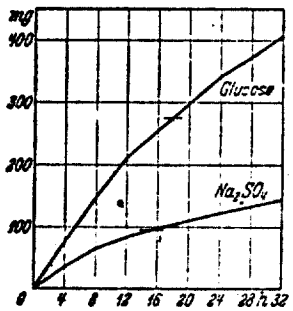

Abb. 4.
Diffusion von isotonischer Glucose- und Na_2SO_4-Lösung durch *Schleicher & Schüll*-Membran.

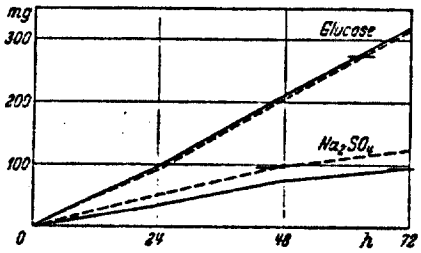

Abb. 5.
Diffusion von isotonischen Dextrose- und Na_2SO_4-Lösungen durch Doppelmembran. (Gestrichelte Linie Mittelschicht dest. H_2O, und ausgezogene Linie Mittelschicht dialysiertes Eiweiß.)

in verschiedenen Variationen (getrocknet, mit Alkohol behandelt usw.) zeigten immer dasselbe. Um ein Analogon zu den Verhältnissen im Körper zu haben, wo der Verlauf gerade umgekehrt ist, wurde dann eine Doppelmembran angewandt mit der folgenden Anordnung: Außen war Aqua dest., innen Glucose-Na_2SO_4 in isotonischen Mengen. Zwischen den beiden Membranen, die *Schleicher & Schüll*sche Pergamentdialysierhülsen verschiedenen Kalibers (40 bzw. 16 mm) waren, wurden nun verschiedene Substanzen gebracht. Die Diffusion erfolgte durch diese, man hatte also eine dicke Membran aus beliebiger Flüssigkeit vor sich. War diese Zwischenflüssigkeit H_2O, so blieben die Verhältnisse dieselben wie bei der einfachen Membran. War die Zwischenflüssigkeit aber z. B. dialysiertes Eiereiweiß, so verlief die Diffusion nun so, wie es Abb. 5 zeigt. Das Na_2SO_4 diffundierte nun langsamer als die Glucose. Die Erklärung ist die, daß Na_2SO_4 nun in dem Eiweiß zurückgehalten wurde, wohl deshalb, weil es mit den Eiweißkörpern in Verbindung trat, während die Glucose diese frei passierte. Das Modell zeigt also dieselben Verhältnisse wie der lebende Darm. Wenn eine diffundierende Substanz von der Membran zurückgehalten wird, so kommt dadurch eine Umkehr der Diffusion zustande. Die Ursachen der selektiven Glucoseresorption im lebenden sind allerdings ganz andere. Immerhin zeigt das Modell, daß man auch mit nichtlebenden Membranen Ähnliches wie mit lebenden erhalten kann.

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STUDIES ON THE STIMULATION BY AMMONIUM SULPHATE OF THE DNA-DEPENDENT RNA POLYMERASE OF ISOLATED RAT-LIVER NUCLEI

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SUMMARY

1. The stimulation by ammonium sulphate of the DNA-dependent RNA polymerase (nucleoside triphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) of isolated rat-liver nuclei has been investigated. Using a preincubation technique, it was possible to obtain experimental conditions in which incorporation of [^{14}C]ATP into RNA was essentially dependent on the presence of the salt.

2. The optimal incubation conditions have been determined for the reaction in the presence and absence of ammonium sulphate, and differences observed in the activation by Mg^{2+} and Mn^{2+}, the pH optima, and the effects of NaF and cysteine. Under both conditions the reaction was DNA-dependent and dependent on the presence of all four nucleoside triphosphates, but differed in its sensitivity to actinomycin D.

3. The RNA products of the reaction in the presence and absence of the salt were found to have different base compositions, different nearest neighbour base frequencies for AMP and GMP, and different sedimentation characteristics on sucrose density gradients.

4. It is inferred from these results that nuclei incubated in the absence of ammonium sulphate may synthesize ribosomal RNA, and that the action of ammonium sulphate is to induce the synthesis of an RNA more DNA-like in its base composition.

INTRODUCTION

The investigation of DNA-dependent RNA polymerase (nucleoside triphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) in extracts of nucleated cells has generally been carried out using a deoxyribonucleoprotein "aggregate enzyme" (WEISS[1], HUANG, MAHESHWARI AND BONNER[2], GOLDBERG[3], BISWAS AND ABRAMS[4]). Although soluble preparations have been obtained from chick embryos (FURTH AND LOH[5]), rat testis (BALLARD AND WILLIAMS-ASHMAN[6]), rat-liver nuclei isolated in

Sulf

2.2 M sucrose (RAMUZ *et al.*[7]) and from bovine lymphosarcoma (FURTH AND HO[8]), none of these authors have reported the solubilization of the fraction of the enzyme activity which is recovered firmly bound to DNA.

In the course of our studies on hormonal effects on nuclear RNA synthesis (TATA AND WIDNELL[9], WIDNELL AND TATA[10]) we have investigated some of the properties of the DNA-dependent RNA polymerase activity of isolated rat-liver nuclei. In order to attempt a complete characterization of the polymerase reaction, it was necessary to isolate a soluble enzyme fraction which is free from the endogenous DNA (HURWITZ *et al.*[11], CHAMBERLIN AND BERG[12], FOX AND WEISS[13], STEVENS AND HENRY[14]). The original aim of the work to be reported here was to obtain such a soluble enzyme preparation from isolated rat-liver nuclei. Several methods designed to achieve this were unsuccessful (WIDNELL[15]), and therefore the stimulation of the enzyme by ammonium sulphate (GOLDBERG[3]) was investigated, since it was considered possible that the stimulation might be caused by solubilization of the enzyme. Although no soluble RNA polymerase could be detected following treatment with ammonium sulphate, we have found that marked differences could be observed when the reaction was assayed in the presence and absence of the salt. Preliminary communications of some of these results have already been presented (WIDNELL AND TATA[16], WIDNELL[17]).

EXPERIMENTAL

Materials

ATP, GTP, CTP, UTP, GDP, CDP, UDP, deoxyribonuclease (EC 3.1.4.5) and ribonuclease (EC 2.7.7.16) were obtained from the Sigma Chemical Co., St. Louis, Mo., U.S.A. ADP was from Boehringer and Soehne GmbH, Mannheim, Germany; dicyclohexylcarbodiimide was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., England; hydracyclonitrile was from Kodak, Ltd., London, England; isopropylidine adenosine and guanosine were from the Aldrich Chemical Co., Inc., Milwaukee, Wisc., U.S.A.; [8-^{14}C]ATP, [8-^{14}C]GTP, [2-^{14}C]CTP and [2-^{14}C]UTP were from Schwartz BioResearch Inc., Orangeburg, N.Y., U.S.A.; Norit A, N.F. IX was from Pfanstiel Laboratories Inc., Waukegan, Ill., U.S.A.; sodium dodecylsulphate was from Matheson, Coleman and Bell, Inc., East Rutherford, N. J., U.S.A.; dried brewers yeast, supplied as readily autolysable strains, type 2 or 1715, was from Phillips Yeast Products Ltd., London, N.W. 10, England. Crude bentonite was obtained from the Amend Drug and Chemical Co., New York, 10, N.Y., U.S.A., and was purified by the method of FRAENKEL-CONRAT, SINGER AND TSUGITA[18].

[α-^{32}P]AMP and [α-^{32}P]GMP were prepared by the method of TENER[19], except that the final chromatographic purification was omitted, and the nucleoside monophosphates converted to [α-^{32}P]ATP and GTP as described by WEISS[1] and NAKAMOTO, FOX AND WEISS[20]. [α-^{32}P]ATP and [α-^{32}P]GTP were purified by adsorption on charcoal (TSUBOI AND PRICE[21]) and, on examination by chromatography on polymin-P-treated paper (RANDERATH[22]) were found to have a radiochemical purity of about 90 %.

Methods

The techniques for the isolation of rat-liver nuclei, and for the assay of DNA have been described previously (WIDNELL AND TATA[23]).

The final procedure adopted for the assay of the RNA polymerase reaction in the absence of ammonium sulphate was as follows: the incubation medium contained, in 0.5 ml: 50 μmoles Tris–HCl buffer (pH 8.5), 2.5 μmoles of $MgCl_2$, 10 μmoles of cysteine, 3 μmoles of NaF; 0.3 μmole each of GTP, CTP and UTP, 0.015 μmole of non-radioactive ATP, 0.005 μmole of [^{14}C]ATP, 0.1 ml of nuclear suspension. After incubation for 15 min at 37°, the RNA was precipitated and extracted as described previously (WIDNELL AND TATA[23]).

The final procedure for the assay of the RNA polymerase reaction in the presence of ammonium sulphate was as follows: the incubation medium contained in 0.5 ml: 50 μmoles of Tris–HCl buffer (pH 7.5), 2 μmoles of $MnCl_2$, 0.05 ml of a solution of ammonium sulphate saturated at room temperature and adjusted to pH 7.5 with NH_4OH, 0.3 μmole each of GTP, CTP, UTP, 0.015 μmole of non-radioactive ATP, and 0.1 ml of nuclear suspension. After preincubation for 15 min at 37°, 0.005 μmole of [^{14}C]ATP was added (0.025 ml of a 0.2 mM solution) and incubation was continued for a further 45 min, after which time the RNA was precipitated and extracted.

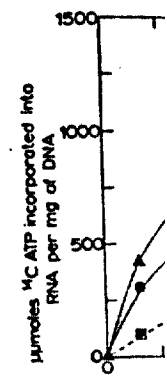
Determination of the nearest neighbour base frequencies and the base composition of the RNA products. The incubation media for the assays were modified as described in the relevant tables. After incubation, the nucleic acids were extracted as described for the routine assay, and then washed twice with 0.2 M perchloric acid and once with abs. ethanol; the RNA was hydrolysed by treatment with 0.3 M KOH for 16 h at 37°, after which the mononucleotides were separated by the method of KATZ AND COMB[24].

Sucrose density-gradient analysis of the RNA products. This was carried out by the method of DALGARNO *et al.*[25]. After incubation, RNA was extracted with 5 % sodium dodecylsulphate, in the presence of bentonite, and layered on a 5–20 % discontinuous sucrose gradient (CHARLWOOD[26]) and centrifuged for 16 h at 22 500 rev./min in the SW-25 rotor of a Spinco preparative ultracentrifuge. Samples were assayed for absorbance at 260 mμ and for acid-insoluble radioactivity as described by DALGARNO *et al.*[25].

RESULTS

The kinetics of the RNA polymerase reaction in the presence and absence of ammonium sulphate

Fig. 1 shows the incubation time course of RNA polymerase of isolated rat-liver nuclei in the presence (Curve B) and absence (Curve A) of ammonium sulphate. When the incorporation of [^{14}C]ATP into RNA due to ammonium sulphate, calculated by subtracting the incorporation in the absence of the salt from the incorporation in its presence, was plotted on the same graph, a straight line passing through the origin was obtained. It was decuced from this result that kinetic differences might exist between the incorporation in the presence and absence of ammonium sulphate. Experiments were therefore designed to determine whether it was possible to obtain



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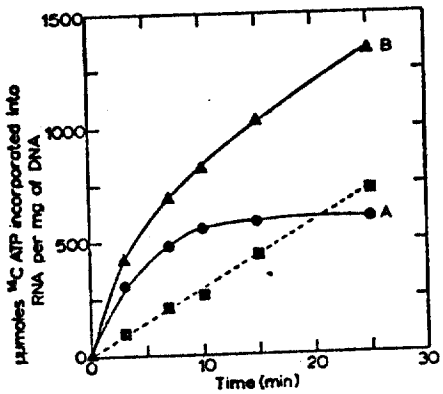

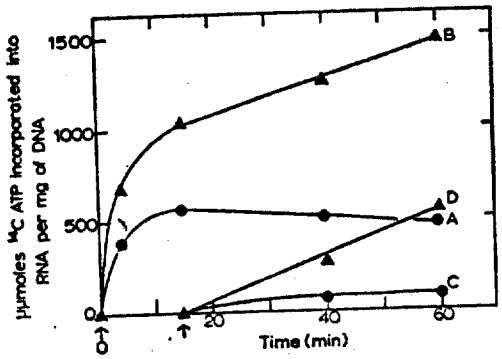

Fig. 1. The effect of ammonium sulphate on the activity of RNA polymerase in isolated liver nuclei. The incubation medium for the assay of RNA polymerase was as described in the text for the Mg^{2+}-activated reaction; ammonium sulphate, adjusted to pH 8.5, was added to a final concentration of 5 % saturated. The continuous line denotes the time course of the reaction in the absence (A) and presence (B) of ammonium sulphate. The broken line denotes the incorporation dependent on the presence of ammonium sulphate, calculated by subtracting the values in Curve A from those in Curve B.

Fig. 2. The kinetics of the RNA polymerase reaction of isolated liver nuclei in the presence and absence of ammonium sulphate. Experimental conditions were as described for Fig. 1. Curves B and D denote the time course of the reaction in the presence of 5 % saturated ammonium sulphate, and Curves A and C, in its absence. [^{14}C]ATP was added (arrows) at time zero for Curves A and B, and after 15 min preincubation in the presence of all four non-radioactive nucleoside triphosphates for Curves C and D.

experimental conditions in which incorporation was essentially dependent on the presence of the salt.

This was achieved by the preincubation technique described in Fig. 2. Curves A and B of Fig. 2 show the incubation time course in the absence and presence of ammonium sulphate, when [^{14}C]ATP was added to the reaction mixture at time zero. Curves C and D show the same for incubations in which [^{14}C]ATP was added after 15 min pre-incubation in the presence of all four non-radioactive nucleoside triphosphates, and the reaction continued for a further 45 min. Under these conditions the incorporation in the absence of ammonium sulphate was only 15 % of that in its presence. It was therefore decided to investigate the reaction in the presence and absence of the salt in greater detail, to determine if differences could be detected between incorporation of [^{14}C]ATP into RNA in the absence of ammonium sulphate, and, after 15 min pre-incubation, in the presence of ammonium sulphate.

Determination of the optimal incubation conditions in the presence and absence of ammonium sulphate

Fig. 3a shows the effect of the concentration of Mg^{2+} and Mn^{2+} in the incubation medium on the polymerase reaction carried out in the presence of ammonium sulphate. At its optimal concentration, Mn^{2+} was 50 % more effective than Mg^{2+}. In the absence of ammonium sulphate however, Mn^{2+} was 20 % less effective than

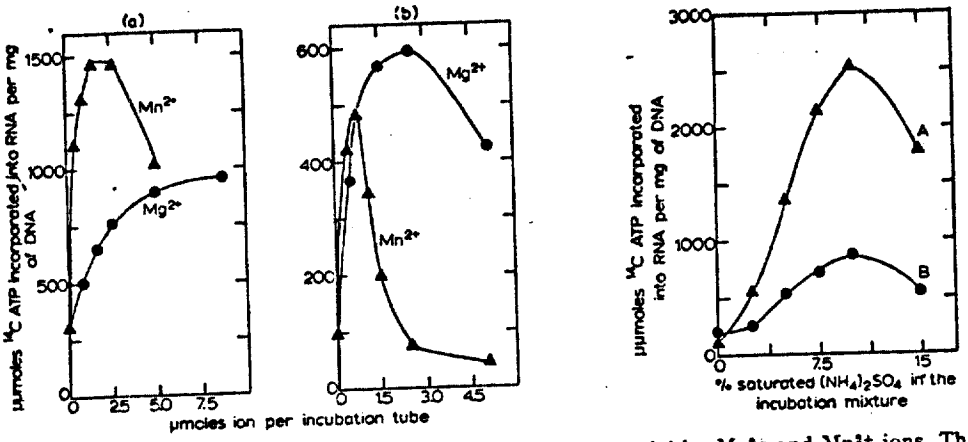

Fig. 3. Activation of the RNA polymerase of isolated liver nuclei by Mg^{2+} and Mn^{2+} ions. The incubation medium for the assay of RNA polymerase was as described for Fig. 1. ●–●, Mg^{2+}; ▲–▲, Mn^{2+}. The nuclear suspension itself contained 0.1 μmole Mg^{2+}. (a) Incubation in the presence of 5 % saturated ammonium sulphate; [^{14}C]ATP was added after 15 min preincubation, and the reaction stopped at 60 min. (b) Incubation in the absence of ammonium sulphate; [^{14}C]ATP was added at time zero and the reaction stopped at 15 min.

Fig. 4. The effect of the concentration of ammonium sulphate on the RNA polymerase reaction of isolated liver nuclei. The incubation conditions were as described in the text. ▲–▲, reaction in the presence of 2.0 μmoles Mn^{2+}; ●–●, reaction in the presence of 2.5 μmoles Mg^{2+}.

Mg^{2+} at its optimal concentration (Fig. 3b). The results presented in Fig. 3b are in good agreement with those of WEISS[1] on the effect of these ions on rat-liver RNA polymerase.

The optimal concentration of ammonium sulphate was 10 % saturated, whether the reaction was activated by Mn^{2+} or Mg^{2+} (Fig. 4). The effect of pH on the reaction in the presence or absence of ammonium sulphate is shown in Figs. 5a and b. The pH optima, 7.5 in the presence of ammonium sulphate, and 8.5 in its absence, were the same whether the reaction was activated by Mg^{2+} or Mn^{2+}. Replacing Tris–HCl with Tris–acetate as the buffer gave essentially the same result, except that under both conditions the pH optimum was slightly elevated. The pH of the incubation medium was not measured and it is possible that this shift only reflects the different characteristics of the two buffers.

Further differences in the optimal incubation conditions for the reaction in the presence of ammonium sulphate were observed when the effects of the concentrations of fluoride ions and cysteine were studied. In the presence of ammonium sulphate fluoride ions had no effect; cysteine was without effect when the reaction was activated by Mg^{2+}, but inhibited slightly in the presence of Mn^{2+}; presumably by chelating the ion (see LENZ AND MARTELL[27]). In the absence of ammonium sulphate, low concentrations of fluoride ions and cysteine stimulated the reaction slightly, and the optimal concentrations were subsequently used in the routine assay.

Table I clearly shows that under both reaction conditions, the incorporation of [^{14}C]ATP into RNA was dependent on the presence of all four nucleoside triphosphates as well as the inhibition by deoxyribonuclease and pyrophosphate. Replacement of the non-radioactive nucleoside triphosphates by the diphosphates caused a marked

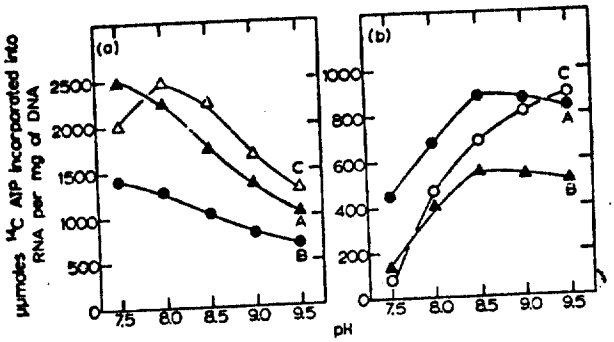

Fig. 5. The effect of pH on the RNA polymerase reaction of isolated liver nuclei, carried out in the presence and absence of ammonium sulphate. Incubation conditions for the reactions were as described in the text. (a) The reaction was carried out in the presence of 10 % saturated ammonium sulphate, adjusted to the pH of the buffer. Reaction mixture contained Tris–HCl buffer and 2 μmoles $MnCl_2$ (Curve A) or 10 μmoles $MgCl_2$ (Curve B). For curve C, the reaction mixture contained Tris–acetate buffer and 2 μmoles $MnCl_2$. (b) The reaction was carried out in the absence of ammonium sulphate. Reaction mixture contained Tris–HCl buffer and 2.5 μmoles $MgCl_2$ (Curve A) or 0.75 μmole $MnCl_2$. For Curve C, the reaction mixture contained Tris–acetate buffer and 2.5 μmoles $MgCl_2$.

TABLE I

THE CHARACTERISTICS OF THE RNA POLYMERASE REACTION OF ISOLATED RAT-LIVER NUCLEI, CARRIED OUT IN THE PRESENCE AND ABSENCE OF AMMONIUM SULPHATE

Incubation conditions were as described in the text.

Incubation medium	*μμmoles of [¹⁴C]ATP incorporated into RNA per mg of DNA*	
	Mg^{2+}	*Mn^{2+}/ ammonium sulphate*
Complete	1034	2980
GTP, CTP and UTP omitted	41	97
CTP and GTP omitted	123	201
CTP and UTP omitted	137	262
UTP and GTP omitted	152	268
GTP omitted	196	366
CTP omitted	192	342
UTP omitted	181	360
GTP, CTP and UTP replaced by GDP, CDP and UDP	463	1045
Complete, + 5 μmoles sodium pyrophosphate	75	37
Complete, + 10 μmoles sodium pyrophosphate	32	23
Complete, + 5 μmoles phosphoenolpyruvate, + 10 μg pyruvate kinase	1109	2893
Complete, deoxyribonuclease (25 μg) at time zero	197	
Complete, ammonium sulphate at 15 min		2140
Complete, deoxyribonuclease (25 μg) at time zero, ammonium sulphate at 15 min		107

reduction in activity, and the ability of nuclei to carry out phosphorylation (PENNIALL, SAUNDERS AND LIU[28]) might explain the residual incorporation. The absence of

any stimulation of the RNA polymerase reaction by phosphoenolpyruvate and pyruvate kinase reflects a low ATPase activity in the nuclear preparations.

Table II shows the effect of salts other than ammonium sulphate on the polymerase reaction activated by Mn^{2+} in the presence of high ionic strength. It can be seen that all the salts tested induced some activation with the exception of

TABLE II

EFFECT OF SALTS OTHER THAN AMMONIUM SULPHATE ON THE RNA POLYMERASE REACTION OF ISOLATED RAT-LIVER NUCLEI ACTIVATED BY Mn^{2+} IN A MEDIUM OF HIGH IONIC STRENGTH

For each incubation the final salt concentration was 0.4 M, and the conditions of assay were, in all other respects, as described in the text for the reaction in the presence of ammonium sulphate.

Salt	*μμmoles [^{14}C]ATP incorporated into RNA per mg of DNA*
$(NH_4)_2SO_4$	3920
NH_4Cl	2020
Li_2SO_4	532
K_2SO_4	1620
Na_2SO_4	1165
KCl	1505
NaCl	1510
None	397

lithium sulphate, but that the activation in all cases was less than that caused by ammonium sulphate. It is likely that the action of lithium sulphate at this concentration in inhibiting RNA polymerase activity is associated with its ability to solubilize proteins associated with nucleic acids (see CURRY AND HERSH[29]), thus possibly preventing interaction between the enzyme and DNA.

The effect of actinomycin D

Preliminary experiments showed that the RNA polymerase reaction, when activated by Mg^{2+} in the absence of ammonium sulphate, was more sensitive to actinomycin D than the "aggregate enzyme" from HeLa cells, assayed in the presence of ammonium sulphate, described by GOLDBERG AND RABINOWITZ[30]. Since a salt concentration of 0.4 M (10 % saturated ammonium sulphate) has no effect on the binding of actinomycin to DNA (REICH, GOLDBERG AND RABINOWITZ[31]), it was decided to investigate in greater detail the sensitivity of the reaction under the two conditions to actinomycin D. Fig. 6 shows that in the absence of ammonium sulphate the reaction was inhibited by 50 % by actinomycin D at a concentration of 0.1 μg/mg of DNA, whilst 4.6 μg actinomycin D per mg of DNA were required for 50 % inhibition of the reaction, when activated by Mn^{2+} in the presence of ammonium sulphate.

Characterization of the RNA product

Nearest neighbour base frequency analysis. Nearest neighbour base frequency analysis of the product of the RNA polymerase reaction, carried out in the absence

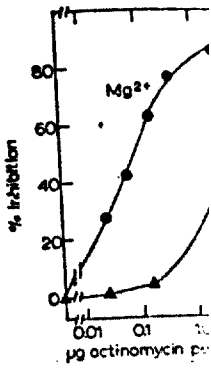

Fig. 6. Effect of act
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TABLE III

NEAREST NEIGHBOU
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Experimental det
cleoside triphosph

Radioactive nucleoside triphosphate	*Inc cond*
[α-^{32}P]ATP	Con me
	Con me
	Co me
	GT UT
	Mg rep Mi
[α-^{32}P]GTP	Co m
	Co m
	C n
	C A
	M r U

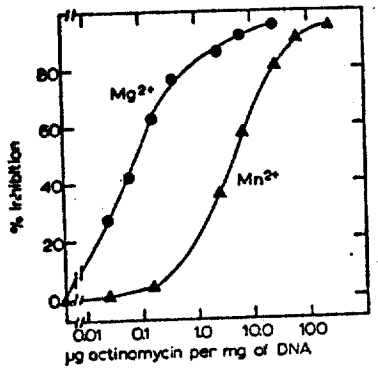

Fig. 6. Effect of actinomycin D on the RNA polymerase reactions of isolated liver nuclei in the presence and absence of ammonium sulphate. Experimental details were as described in the text. Actinomycin D was added at time 0. ●–●, reaction in the absence of ammonium sulphate; ▲–▲, reaction in the presence of ammonium sulphate.

TABLE III

NEAREST NEIGHBOUR BASE FREQUENCY ANALYSIS OF THE PRODUCT OF THE RNA POLYMERASE REACTION OF ISOLATED RAT-LIVER NUCLEI CARRIED OUT IN THE ABSENCE OF AMMONIUM SULPHATE

Experimental details were as described in the text, except that the concentration of each nucleoside triphosphate in the reaction mixture was 0.3 µmole.

Radioactive nucleoside triphosphate	*Incubation conditions*	*Experiment*	*Per cent of total ^{32}P recovered in*				*^{32}P input (counts/min)*	*^{32}P recovered (counts/min)*
			UMP	*GMP*	*CMP*	*AMP*		
[α-^{32}P]ATP	Complete medium	1	19	33	28	20	7850	7760
	Complete medium	2	20	29	29	22	3900	3850
	Complete medium	3	22	30	27	21	11 750	11 100
	GTP, CTP, UTP omitted	3	19	32	28	21	1950	1835
	Mg^{2+} replaced by Mn^{2+}	3	17	34	31	18	9350	8750
[α-^{32}P]GTP	Complete medium	1	19	34	33	14	12 300	12 350
	Complete medium	2	19	34	30	17	7100	6970
	Complete medium	3	18	33	35	14	18 750	18 100
	CTP, UTP, ATP omitted	3	20	33	30	17	1560	1645
	Mg^{2+} replaced by Mn^{2+}	3	17	35	30	18	14 150	13 700

of ammonium sulphate is presented in Table III. In the RNA synthesized under these conditions, both AMP and GMP were adjacent to GMP and CMP more often than they were adjacent to UMP and AMP. This nearest neighbour frequency was unaltered when Mn^{2+} replaced Mg^{2+} in the incubation medium, or when the non-radioactive nucleoside triphosphates were omitted. The low incorporation in the absence of added non-radioactive nucleoside triphosphates could be due to a very small pool of these compounds in the isolated nuclei. The nearest neighbour analysis of the RNA product of the polymerase reaction carried out in the presence of ammonium sulphate is reported in Table IV. Under these conditions also, the nearest

TABLE IV

NEAREST NEIGHBOUR BASE FREQUENCY ANALYSIS OF THE PRODUCT OF THE RNA POLYMERASE REACTION OF ISOLATED RAT-LIVER NUCLEI CARRIED OUT IN THE PRESENCE OF AMMONIUM SULPHATE

Experimental details were as described in the text, except that the final concentration of each nucleoside triphosphate was 0.3 μmole.

*Radioactive nucleoside triphosphate**	*Incubation conditions*	*Experiment*	*Per cent of total ^{32}P counts/min reovered in*				*Total ^{32}P on column (counts/min)*	*^{32}P recovered (counts/min)*
			UMP	*GMP*	*CMP*	*AMP*		
[α-^{32}P]ATP	Complete medium	1	25	26	20	29	9730	9540
	Complete medium	2	26	24	20	30	7300	7080
	Complete medium	3	24	26	21	29	16 450	15 150
	GTP, CTP, UTP omitted	3	23	23	22	32	1470	1495
	Mn^{2+} replaced by Mg^{2+}	3	28	25	19	28	11 050	11 250
[α-^{32}P]GTP	Complete medium	1	37	25	13	25	8860	8490
	Complete medium	2	34	27	10	29	4750	4810
	Complete medium	3	35	26	12	27	14 800	15 020
	CTP, UTP, ATP omitted	3	37	24	9	30	1390	1280
	Mn^{2+} replaced by Mg^{2+}	3	31	30	9	30	9900	9750

* 0.15 μmole of the radioactive nucleoside triphosphate was added after 15 min preincubation in the presence of all four non-radioactive triphosphates.

neighbour frequency was unaltered when Mg^{2+} replaced Mn^{2+} in the incubation medium, or when the non-radioactive nucleoside triphosphates were omitted. However, the presence of ammonium sulphate caused a marked change in the nearest

neighbour base frequency in that the relative occurrence of nucleotides adjacent to AMP was AMP > UMP = GMP > CMP and adjacent to GMP: UMP > AMP > GMP > CMP.

Base composition analysis. The base composition of the RNA synthesized in the two reactions determined by the incorporation of all four ^{14}C-labelled nucleoside triphosphates is shown in Table V. It can be seen that the RNA product of the polymerase reaction, activated by Mg^{2+} in the absence of ammonium sulphate, had a

TABLE V

THE BASE COMPOSITION, DETERMINED BY THE INCORPORATION OF [^{14}C]ATP, GTP, CTP AND UTP, OF THE RNA SYNTHESIZED BY THE RNA POLYMERASE REACTION OF ISOLATED RAT-LIVER NUCLEI IN THE PRESENCE AND ABSENCE OF AMMONIUM SULPHATE

Experimental conditions were as described in the text, except that the concentrations of nucleoside triphosphates were as follows: in the absence of ammonium sulphate: μmmoles of each nucleotide per incubation: Expt. 1, 100; Expt. 2, 10; Expt. 3, 20. In the presence of ammonium sulphate: Expt. 1, 100; Expt. 2, 5; Expt. 3, 10. Preincubation was in the absence of any nucleoside triphosphate.

Incubation conditions	*Experiment*	*μmoles of nucleotide per 100 μmoles of RNA**				*Total counts on column*	*Counts recovered*
		UMP	*GMP*	*CMP*	*AMP*		
Mg^{2+} activated in the absence of ammonium sulphate	1	18	31	31	20	29 300	27 900
	2	20	33	30	17	4440	4370
	3	19	35	29	17	7350	7420
Mn^{2+} activated in the presence of ammonium sulphate	1	25	25	23	27	33 500	29 800
	1, ammonium sulphate omitted	18	32	29	21	1890	1910
	2	23	27	24	26	2050	2090
	3	24	26	23	27	3780	3770

* The concentration of each nucleotide in the product was calculated by dividing the radioactivity recovered in each by the specific activity of the original nucleoside triphosphate.

higher content of GMP and CMP than of UMP and AMP, and that the RNA synthesized in the presence of ammonium sulphate had approximately the same content of all four nucleotides. The experiment in which ammonium sulphate was omitted from the RNA polymerase reaction activated by Mn^{2+} demonstrated that the change in the base composition was caused by the presence of ammonium sulphate, irrespective of the presence of Mg^{2+} or Mn^{2+}.

Sucrose density-gradient analysis of the RNA products. To obtain decisive results with sucrose gradient centrifugation, it was necessary to have RNA of higher radioactivity than that of the other experiments. To achieve this, incubation was carried out in the presence of both [α-^{32}P]ATP and [α-^{32}P]GTP, and the product after 1 min incubation was compared with that after 15 min. It can be seen (Fig. 7a) that when the reaction was activated by Mg^{2+} in the absence of ammonium sulphate, a greater proportion of the labelled RNA obtained after 1 min of incubation was of high molecular weight, sedimenting as 20–50-S RNA, than that obtained after 15 min of incubation; and that the addition of ammonium sulphate at the end of the incubation, immediately before the extraction of RNA, had no effect on the sedi-

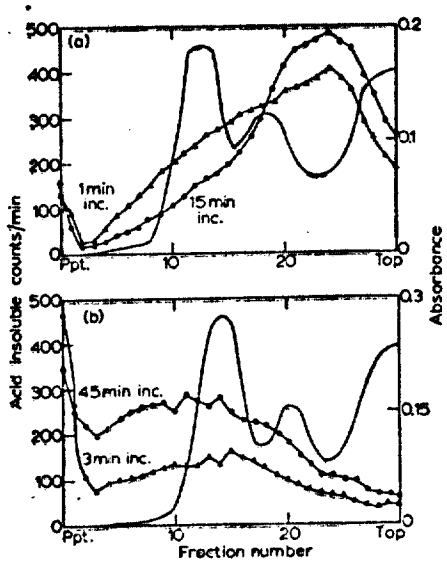

Fig. 7. Sucrose density-gradient centrifugation analysis of the RNA products. (a) The product of the reaction in the absence of ammonium sulphate. For the 1-min incubation, the medium contained 0.3 μmole each of [α-^{32}P]ATP and [α-^{32}P]GTP and for the 15-min incubation, 0.05 μmole of each. ▲–▲, RNA product of 1 min incubation; ●–●, RNA product of 15 min incubation. (b) The product of the reaction in the presence of ammonium sulphate. For the 3-min incubation, 0.2 μmole each of [α-^{32}P]ATP and [α-^{32}P]GTP were added after 15 min preincubation and for the 45-min incubation 0.05 μmole of each. ▲–▲, RNA product of 3 min incubation; ●–●, RNA product of 45 min incubation. Other experimental details were as described in the text. The continuous line denotes the profile of RNA (absorbance at 260 mμ) from reticulocyte ribosomes added as a marker.

mentation profile. No RNA with an S value greater than about 50 was found at any time interval of incubation, apart from the small fraction recovered in the pellet and in Fraction 1. Fig. 7b shows that a higher proportion of the RNA product of the polymerase reaction activated by Mn^{2+} in the presence of ammonium sulphate, sedimented to the bottom of the tube after 3 min of incubation than after 45 min. The majority of the RNA obtained in the pellet was again recovered in the pellet following resuspension and resedimentation in a sucrose density gradient. Although the results in Fig. 7 suggest that the RNA product was degraded during incubation, it is unlikely that the marked differences observed in the sedimentation profiles of the products of the reaction under the two conditions could be explained entirely by different rates of degradation.

Although the products of the reaction under the two incubation conditions had different nearest neighbour base frequencies and base compositions, it was considered possible that similar RNA fractions might be present in the product of each reaction. It was therefore decided to investigate the two products in greater detail, by comparing the distribution of radioactivity in mononucleotides obtained by alkaline hydrolysis of RNA fractions with different sedimentation coefficients. RNA, labelled with [α-^{32}P]ATP and [α-^{32}P]GTP, was subjected to sucrose density-gradient centrifugation as described in Figs. 10a and b; fractions were collected and the mononucleotides separated after alkaline hydrolysis. The percentage of the radioactivity recovered in each mononucleotide is shown in Table VI, where it can be seen that for

TABLE VI

THE DISTRIBUTION OF RADIOACTIVITY IN MONONUCLEOTIDES OBTAINED AFTER ALKALINE HYDROLYSIS OF RNA FRACTIONS RECOVERED FROM SUCROSE DENSITY GRADIENTS

RNA samples were obtained as described in the text, and fractions were taken from gradients identical to those described in Figs. 10a and b.

RNA sample	*Fraction No.*	*Per cent of total ^{32}P counts/min recovered in*			
		UMP	*GMP*	*CMP*	*AMP*
Product of the reaction in the absence of ammonium sulphate	Precipitate	18	34	30	18
	6–14	14	36	32	18
	22–27	17	33	30	20
Product of the reaction in the presence of ammonium sulphate	Precipitate	27	27	20	26
	1	26	27	20	27
	2–14	28	26	20	26
	17–27	29	27	17	27

RNA synthesized in the same reaction, the distribution of radioactivity between the mononucleotides was essentially the same in fractions with different sedimentation coefficients.

DISCUSSION

The results presented here show that the presence of ammonium sulphate caused marked changes in the characteristics of the DNA-dependent RNA polymerase of isolated rat-liver nuclei. Both in the presence and absence of the salt the reaction was DNA-dependent and dependent on the presence of all four nucleoside triphosphates. However, in the absence of the salt the reaction was preferentially activated by Mg^{2+}, and in the presence of the salt the reaction was preferentially activated by Mn^{2+}. When the reaction under the two conditions was investigated further, it was found that there were differences in the optimal incubation conditions, in the sensitivity to actinomycin D, and that the RNA products had different nearest neighbour base frequencies, base compositions and sedimentation characteristics.

It is interesting to note that the pH optima and activation by manganese and magnesium ions observed here for the reaction in the presence of ammonium sulphate are similar to those reported for soluble RNA polymerase preparations from bacterial sources (CHAMBERLIN AND BERG[32]; FURTH, HURWITZ AND ANDERS[33]; FOX AND WEISS[13]), from rat testis (BALLARD AND WILLIAMS-ASHMAN[6]), from rat liver (RAMUZ *et al.*[7]) and from bovine lymphosarcoma (FURTH AND HO[8]).

The nearest neighbour base frequency analysis and base composition studies suggest that the RNA synthesized in the absence of ammonium sulphate was similar in composition to ribosomal RNA, and the results are in good agreement with those of HURLBERT, LIAU AND ORENGO[31]. The RNA synthesized in the presence of the salt was more DNA-like (for the base composition of rat-liver DNA and ribosomal RNA, see MUNRO AND KORNER[35]), and it is interesting to note that the base composition of the RNA synthesized in the presence of ammonium sulphate was essentially

the same as that of rapidly labelled rat-liver cytoplasmic RNA (MUNRO AND KORNER[35]). The finding that the reaction was more sensitive to actinomycin D when carried out in the absence of ammonium sulphate is in general agreement with results obtained *in vivo*, where it has been found that low doses of actinomycin preferentially inhibited the synthesis of ribosomal RNA (PERRY[36]; TRAKATELLIS, AXELROD AND MONTJAR[37]).

The results of sucrose density-gradient analysis show that the RNA product synthesized in the absence of ammonium sulphate was of lower molecular weight than that synthesized in its presence. The distribution of radioactivity in mononucleotides obtained by hydrolysis of RNA fractions with different sedimentation coefficients was essentially the same, and was consistent with the degradation of the product during incubation; it does, however, indicate a consistency in the RNA product under each reaction condition. The observation that no RNA with a sedimentation constant greater than about 50 S was synthesized in the absence of ammonium sulphate is in general agreement with the results of SCHERRER, LATHAM AND DARNELL[38] and RAKE AND GRAHAM[39] who have shown precursors of ribosomal RNA of about that size in HeLa and L cells. HUANG, BONNER AND MURRAY[40] have shown that an ionic strength of 0.4 or greater is sufficient to solubilize histones from DNA, and the high sedimentation constant of the RNA synthesized in the presence of ammonium sulphate would be consistent with its formation on a large unobstructed template.

These results of HUANG *et al.*[40], together with those of CHAMBON, RAMUZ AND DOLY[41], who showed that ammonium sulphate stimulated RNA synthesis by a DNA–RNA polymerase–histone complex, could suggest that the action of ammonium sulphate might only be to remove histones from DNA. It is, however, difficult to understand (a) how removal of histones from DNA could alter the pH optimum and ionic activation of the reaction, and (b) why nuclei, which presumably were capable of synthesizing DNA-like RNA in the intact cell, should apparently lose this capacity except in the presence of ammonium sulphate, during the process of isolation.

In view of the evidence that ribosomal RNA is synthesized in the nucleolus (PERRY[36]; BROWN AND GURDON[42]; MCCONKEY AND HOPKINS[43]; LIAU, HNILICA AND HURLBERT[44]), it would be of interest to determine whether the reaction in the absence of ammonium sulphate represents nucleolar RNA synthesis, and whether the reaction in the presence of the salt represents chromosomal RNA synthesis. Although DNA-dependent RNA polymerase activity has been detected in nucleolar preparations (RO, MURAMATSU AND BUSCH[45]; TSUKADA AND LIEBERMANN[46]; LIAU *et al.*[44]), no studies have been presented on the effect of ammonium sulphate on the system.

Although the physiological significance of the observations reported here is unknown, we have found that the activity of the reaction is depressed in nuclei from livers of hypophysectomized, thyroidectomized or castrated rats whether the assay was carried out in the presence or absence of ammonium sulphate. Furthermore, we have also found that the reactions under the two conditions respond independently to thyroid hormone, growth hormone and testosterone (TATA AND WIDNELL[9]; WIDNELL AND TATA[10]), when the hormones were administered at doses which caused an acceleration of the growth of liver.

In conclusion, therefore, nuclei incubated in the absence of ammonium sulphate synthesize RNA with characteristics which suggest that it is ribosomal RNA, and the action of ammonium sulphate is to induce the synthesis of an RNA more DNA-like in its base composition. The complexity of the nucleoprotein responsible

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for RNA polymerase activity in isolated rat-liver nuclei is such that no conclusions may be drawn concerning the mechanism of the action of ammonium sulphate on the system. The results further emphasize the necessity for isolating more purified enzyme preparations for the study of mammalian RNA polymerase.

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SUMMARY

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INTRODUCTION

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Activation of mammalian glycerate dehydrogenase by inorganic salts

The activation or inhibition of various dehydrogenases by inorganic salts has been reported by several groups of investigators. JOYCE AND GRISOLIA[1] observed reactivation of malate dehydrogenase (EC 1.1.1.37) by Tris–Cl^- and by ammonium sulfate. BURTON AND WILSON[2] studied the effect of NaCl or fumarate on this enzyme. They concluded that the observed increase in the equilibrium constant was due to the effect of increased ionic strength on the activity coefficients of the reactants. THEORELL *et al.*[3] have demonstrated either activation or inhibition of liver alcohol dehydrogenase (EC 1.1.1.1) by Cl^-, depending on the concentrations of substrate and coenzyme used. The activity of glucose-6-phosphate dehydrogenase (EC 1.1.1.29) was stimulated as much as 6-fold by low concentrations of various anions, while inhibition resulted at higher concentrations[4]. In experiments with crystalline glyoxylate reductase (EC 1.1.1.26) from tobacco leaves, anion activation of hydroxypyruvate reduction has been reported[5]. HOLZER AND HOLLDORF[6] have demonstrated the activation of hydroxypyruvate reductase from spinach leaves by various anions, in the order $Br^- \gg NO_3^- \gg SO_4^{2-} = Cl^- = I^- \gg F^-$. The effect of various salts on the activity of mammalian glycerate dehydrogenase (D-glycerate: NAD^+ oxidoreductase, EC 1.1.1.29) is reported in this paper. The enzyme catalyzes the reaction:

$$\text{D-glycerate} + NAD^+ \text{ (or } NADP^+) \rightleftharpoons \text{hydroxypyruvate} + H^+ + NADH \text{ (or } NADPH)$$

Since Tris–Cl^- buffers were routinely used in our earlier studies, the effect of Cl^- was not observed until a study of pH optima in various buffers was initiated.

The method described by DICKENS AND WILLIAMSON[10] was used to prepare lithium hydroxypyruvate. NADH was purchased from Pabst Laboratories. The reagent-grade inorganic salts were commercial products. Beef liver glycerate dehydrogenase, free of lactate dehydrogenase (EC 1.1.1.27), was prepared as previously reported[8].

The 2.25 ml incubation medium contained: 0.250 μmole NADH; 1.43 μmoles lithium hydroxypyruvate (omitted from the control); 120 μmoles imidazole–acetate buffer (pH 6.3); 125 μmoles inorganic salt, added as indicated in the figures; and enzyme solution (0.059 mg of protein) added last. As shown in Fig. 1, the reaction rate was linear for at least 5 min. The values plotted are averages of two or more experiments with each salt.

Under the conditions specified the optimal chloride concentration was found to be from 0.056 M to 0.078 M. The final concentration of the salts used in this study was 0.056 M unless stated otherwise. In the first experiment the effect of various anions was determined (Fig. 1). These data indicating the following relationship: $Cl^- = Br^- = I^- \gg NO_3^- \gg F^-$. Results similar to those observed with NaF were obtained upon the addition of sodium or ammonium sulfate (0.028 M) or potassium or sodium phosphate buffer (pH 6.3) (0.042 M $H_2PO_4^-$, 0.014 M HPO_4^{2-}). Various chloride salts were tested at the chloride concentration found to be optimal with sodium as the cation (Fig. 2). Although lower activities were observed with lithium and ammonium chloride, the differences were not as great as those observed with the various anions. The possibility remains that the lower activities which these cations may have been due to impurities in the commercial salts used.

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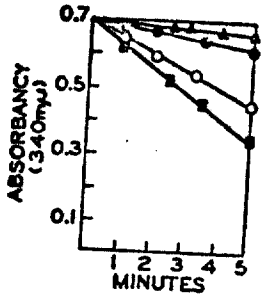

Fig. 1. Anion activation. Additions: ▲, none; ●, NaF; ○, $NaNO_3$; ■, NaCl, NaBr, or NaI.

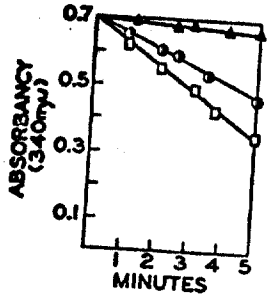

Fig. 2. Various chloride salts as activators. Additions: ▲, none; ●, LiCL or NH_4Cl; □, NaCl or KCL.

The mechanism of the ion activation of glycerate dehydrogenase is unknown. The activation of enzymes by cations and anions has been studied classically through the application of kinetic analysis[11-14]. Further studies of mammalian glycerate dehydrogenase are in progress in order to determine the effect of ions on the kinetic constants.

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Table I

Conversion of stearoyl-1-^{14}C-CoA to sphingomyelin

The incubation mixtures in Part A contained 100 μmoles of potassium phosphate buffer, pH 7.8, 80 mμmoles of stearoyl-1-^{14}C-CoA (67,600 cpm), 2 μmoles of ATP, 2 μmoles of $MgCl_2$, and resuspended rat brain particulate preparations in a final volume of 1.18 ml. In Part B, the components of the mixtures were the same except that 1.0 ml of 100,000 × *g* supernatant solution, obtained by treating the recovered particulate fractions with sodium cholate, was employed. Incubated under an atmosphere of N_2 for 3 hou of the incubation period, 0.1 ml of methanol cont myelin (Wilson Laboratories. Experiment 1; S periment 2) was added to each sample as carri the recovery of the labeled sphingomyelin is

Source of enzyme and additions		
Part A		
Particles from 600 to 8,400 × *g*		
None	1,200	900
Sphingosylphosphorylcholine*	8,375	6,174
Sphingosylphosphorylcholine, boiled control	350	245
Particles from 8,400 to 100,000 × *g*		
None	375	363
Sphingosylphosphorylcholine	8,875	3,185
Spingosylphosphorylcholine, boiled control	100	173
Part B		
Cholate supernatant, 600 to 8,400 × *g* particles		
None	650	600
Spingosylphosphorylcholine	4,375	1,550
Sphingosylphosphorylcholine, boiled control	475	300
Cholate supernatant, 8,400 to 100,000 × *g* particles		
None	325	317
Spingosylphosphorylcholine	400	376
Sphingosylphosphorylcholine, boiled control	225	210

* The amount of sphingosylphosphorylcholine added in each instance was 1 mg.

Table II

Effect of various cofactors on conversion of stearoyl-1-^{14}C-CoA to sphingomyelin

Except as indicated, the incubation mixtures contained 100 μmoles of potassium phosphate buffer, pH 7.0, 80 mμmoles of stearoyl-1-^{14}C-CoA (67,600 cpm), 2 μmoles of ATP, 2 μmoles of $MgCl_2$, and 1.0 ml (6.0 mg of protein) of the 100,000 × *g* supernatant solution obtained by treating the 600 to 8,400 × *g* particles with sodium cholate. The conditions for incubation and recovery of sphingomyelin were the same as in Table I.

Additions	Deletions	Radioactivity of recovered sphingomyelin
		cpm
None	None	589
Sphingosylphosphorylcholine, 2 μmoles	None	1,984
Sphingosylphosphorylcholine, 2 μmoles, psychosine, 2 μmoles	None	1,612
Sphingosylphosphorylcholine, 2 μmoles, sphingosine, 2 μmoles	None	1,550
Sphingosylphosphorylcholine, 2 μmoles	ATP	2,046
Sphingosylphosphorylcholine, 2 μmoles, stearic acid-1-^{14}C, 80 mμmoles (67,500 cpm)	Stearoyl-1-^{14}C-CoA	558
Sphingosylphosphorylcholine, 2 μmoles, boiled control	None	155

son and Blecher (11). After visualizing and eluting the sphingomyelin area, 1087 cpm (94.2%) were recovered.

It appears therefore that the biosynthesis of sphingomyelin in brain may occur either from ceramide and CDP-choline as described by Sribney and Kennedy (1) and supported by the findings of Kopaczyk and Radin (12) *in vivo* or via the acylation of sphingosylphosphorylcholine.

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The Effect of High Concentrations of Salt on Kinetic Properties of Pancreatic Ribonuclease*

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With the exciting developments of the application of x-ray diffraction analysis to the study of protein structure in the crystalline state, the question of the validity of equating the crystal structure with the structure in solution has become a very pressing one (see Reference 1). Many proteins are crystallized from concentrated salt solutions (mostly ammonium sulfate), and in these cases, it is generally possible to evaluate the effect of salt on the structure of the protein in question. The results of several such studies indicate that high concentrations of salt may indeed disrupt the forces that stabilize the tertiary and quaternary structure of proteins. Thus, it has been found that hemoglobin dissociates in concentrated salt solutions, apparently without any further structural changes (2). Rupley (3), studying the effect of salt on methemoglobin, found significant changes in the tyrosine titration in this protein as the salt concentration was increased, indicating that the salt does cause structural alterations. Furthermore, since methemoglobin crystal suspensions and methemoglobin solutions showed very similar properties in concentrated salt solutions, it was concluded that the protein crystallized as the structurally modified form. Recently, Winstead and Wold (4) have reported results which indicate that rabbit muscle enolase dissociates in 2 M ammonium sulfate. In this system, a significant change in the frictional ratio of the protein was observed under conditions (high protein concentration and high salt concentration) favorable to crystallization

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On the other hand, arguments in favor of identical protein structures in solution and in the crystalline state have also been presented. One of the strongest of these is based on the finding of Doscher and Richards (5) that ribonuclease S has full enzymatic activity in the crystalline state in concentrated ammonium sulfate solutions.

This communication reports some preliminary observations on the activity of ribonuclease A and ribonuclease S at high concentrations of salt. The observations have led us to raise the question whether the active form of this enzyme in concentrated salt solutions may not in fact be different from the active form of the enzyme in solutions of low ionic strength.

Bovine pancreatic ribonuclease A was prepared from ribonuclease (Sigma), crystallized five times, by the method of Hirs, Moore, and Stein (6). The enzyme, freed from salt on a Sephadex G-25 column eluted with 50% acetic acid, was lyophilized. The product, which may contain as much as 50% aggregated material (7) after lyophilization, was used without further treatment as "dimer," while ribonuclease A was obtained from the lyophilized material by heating at 65° for 10 min (7). Ribonuclease S was prepared by digestion with Nagarse (bacterial proteinase obtained from the Biddle Sawyer Corporation) according to the method of Gordillo, Vithayathil, and Richards (8), and S-protein and S-peptide were separated on Sephadex G-75 with 50% acetic acid as solvent (7). The reconstituted enzyme, ribonuclease S′, was used in all of this work. Enzymatic activity (measured at 25°) with cytidine 2′,3′-phosphate as substrate was measured in 0.1 M Tris-HCl buffer, pH 7.0, according to the method of Crook, Mathias, and Rabin (9)[1]; with yeast RNA (Eastman Organic Chemicals) as substrate, the assays were carried out in 0.1 M acetate buffer, pH 5.0, according to the rapid method of Anfinsen *et al.* (10).[1] In all of the experiments, the salt was added to the buffered assay mixtures, and the "zero salt" values in the figures thus represent the activity in the above buffers (0.1 M) in the absence of added salt. Protein concentrations were based on dry weight.

The effect of ammonium sulfate on the activity of ribonuclease A is shown in Fig. 1, and it is in general agreement with that reported by Doscher and Richards (5) for ribonuclease S. With cyclic cytidylic acid as the substrate, the activity decreased to a minimum at an ammonium sulfate concentration of 0.5 to 1 M, and then increased again to give full activity in 3 M ammonium sulfate. With yeast RNA as substrate, the activity decreased steadily with salt concentration to give complete inactivation at 2 M salt.

By using only the cyclic cytidylic acid assay, the effect of several salts was tested in comparison to ammonium sulfate, and different forms of the enzyme were also studied. The data in Fig. 1 show that the divalent anions have the strongest effect as inhibitors and also as activators at high concentration, and it is interesting to note that even phosphate causes activation of the enzyme at high enough concentration. Considering that at the pH of the assay, only 50% of the phosphate is present as the

[1] The effect of the high salt concentrations on the spectral properties of cyclic cytidylic acid and CMP and thereby on the rate assay was evaluated and found to be insignificant. Similarly, the effect of ammonium sulfate on the solubility of ribonuclease-digested RNA in the trichloroacetic acid reagent was tested to validate the RNA assay. No significant effect was found at lower salt concentration, but with 2 M ammonium sulfate, there was an increase in nucleotide solubility, so that any activity observed against RNA in 2 M ammonium sulfate would be biased upward.

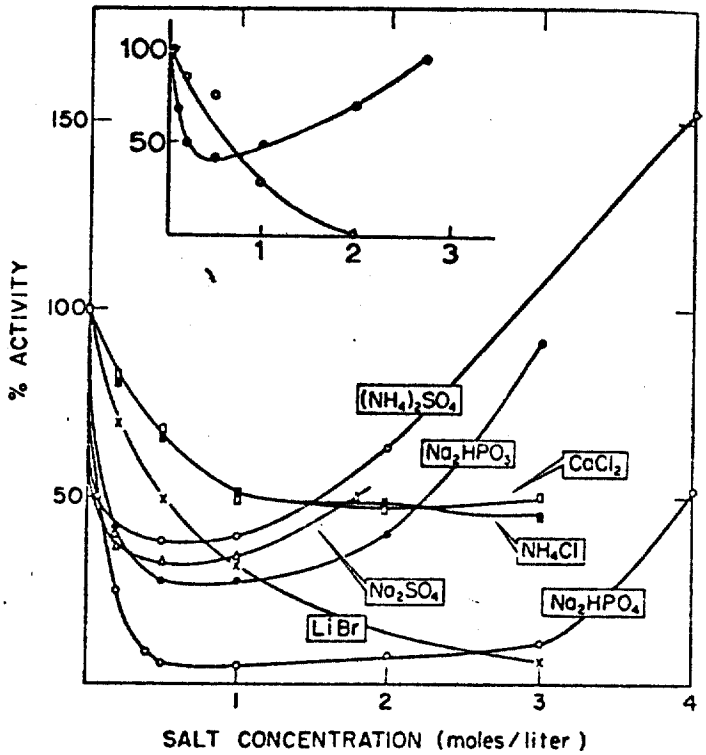

FIG. 1. The effect of salts on ribonuclease A activity. The effect of several salts on the rate of cyclic cytidylic acid hydrolysis by ribonuclease A. In the case of phosphite and phosphate, the salt was a mixture of the monoanions and dianions at the assay pH of 7, and the results are plotted as total concentrations of both species. *Insert*, the effect of ammonium sulfate with RNA (○——○) and with cyclic cytidylic acid (●——●) as substrates.

dianion, phosphate is in fact as effective as sulfate in activating the enzyme.

Fig. 2 shows a comparison of the activities of ribonuclease A, ribonuclease A dimers, ribonuclease S′, and S-protein and S-peptide mixed after salt treatment. The activity of ribonuclease S′ in 3 M ammonium sulfate was always significantly higher than that of ribonuclease A in parallel experiments. It is also interesting to note that S-protein incubated with 3 M ammonium sulfate in the absence of S-peptide was no longer capable of forming active ribonuclease S when S-peptide was added. Under the assay conditions used (in one set of experiments the substrate-salt mixture was activated by addition of ribonuclease S′ and in the other the S-protein-substrate-salt mixture was activated by addition of S-peptide, and in both cases the absorbance changes were followed for several minutes), one must consider the possibility that the observed activity difference is a rate effect, and that prolonged incubation would have eventually given the same activity in both samples. This point should not affect the main argument, however.

The lyophilized sample labeled as ribonuclease A dimer was included to test the possibility that aggregated forms of ribonuclease A might account for the increase in activity at high salt concentration. If such aggregated forms were similar to the aggregates formed during lyophilization from acetic acid, the stimulation of activity by salt might be expected to occur at lower salt concentrations when the dimer was used. Since there was no difference between the lyophilized samples and the lyophilized and heated samples, this mechanism is untenable. It is in fact quite possible that the high salt concentration may dissociate any dimers present in the reaction mixture.

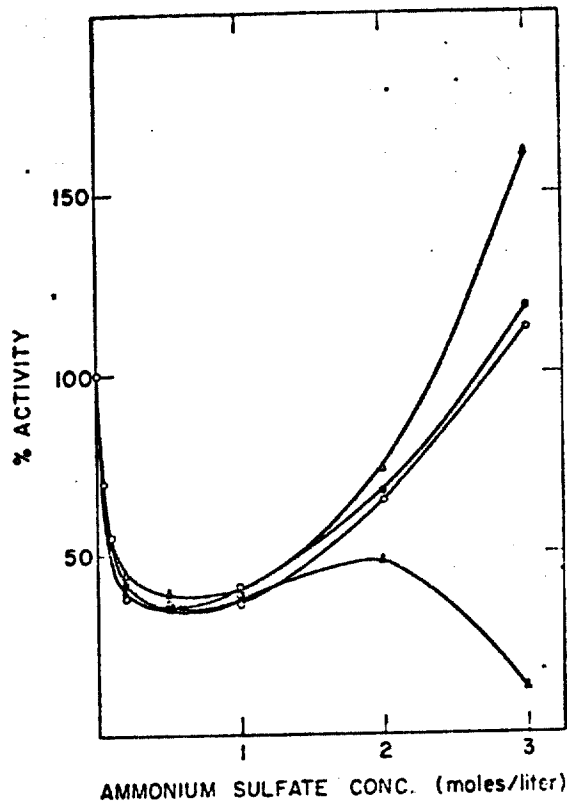

FIG. 2. The effect of ammonium sulfate on the activity of differen forms of ribonuclease. ○——○, ribonuclease A; ●——●, ribonuclease dimer; △——△, ribonuclease S′; ▲——▲, S-protein + S-peptide (S-protein was incubated with salt and substrate, the system was activated by the addition of 2 eq of S-peptide in buffer, and the rate was determined during the first 3 to 4 min).

TABLE I

Relative rates of hydrolysis of cyclic cytidylic acid by ribonuclease A in presence and absence of salt*

	No salt	$(NH_4)_2SO_4$, 3 M	NH_4Cl, 3 M
No inhibitor	100	107	34
+ Phosphate, 0.05 M	29, 21	100	38
+ RNA, 0.1 mg	73, 70	108	33

* The rate of hydrolysis was linear with cyclic cytidylic acid in the concentration range of 0.5 to 3 × 10^{-4} M, and it is reported here in arbitrary relative units.

A very substantial volume of work on the effect of ionic strength and of various specific ions on ribonuclease activity has been reported. Most of the previous work was, however, carried out at salt concentrations below 1 M, and in general under conditions which are not directly comparable to those employed here. We have, for example, found that the effect of salt is sensitive to pH (the inhibition of cyclic cytidylate hydrolysis by low concentration of ammonium sulfate is enhanced and the stimulation of hydrolysis by high concentrations of ammonium sulfate is decreased at pH 5 as compared to the data reported here for pH 7), and it is thus possible that apparent discrepancies such as the stimulatory effect of increased ionic strength up to 0.3 reported by Davis and Allen (11) (using cyclic uridylate), the lack of ionic strength effect in the same range, reported by Hummel, Flores, and Nelson (12) (using cyclic cytidylate), and the inhibition found in our work (Figs. 1 and 2) may be explained by differences in the assay conditions.

In an attempt to compare the enzymatic properties of ribonuclease in the absence and in the presence of salt, the effect of phosphate and RNA as inhibitors of cyclic cytidylic acid cleavage was studied. The results given in Table I shows that the inhibition by both phosphate and RNA which can be observed in the absence of salt was eliminated in high salt, whether it was 3 M ammonium chloride (35% of the original activity remaining) or 3 M ammonium sulfate (100% activity was observed).

There are thus three findings that are consistent with the proposal that ammonium sulfate can interfere with the forces involved in stabilizing the tertiary and quarternary structure of proteins, and that the active form of ribonuclease in concentrated ammonium sulfate solutions is different from the "native" form in dilute salt solutions.

1. The native enzyme hydrolyzes both cyclic cytidylate and RNA in dilute buffer, and the hydrolysis of cyclic cytidylate is inhibited by both RNA and phosphate. In the presence of high concentrations of salt, the rate of hydrolysis of cyclic cytidylate is enhanced, while the rate of RNA hydrolysis is greatly diminished, and the inhibitory effect of both RNA and phosphate is eliminated.

2. While ribonuclease S′ and ribonuclease A show very similar catalytic properties in dilute buffer and at low salt concentrations, ribonuclease S′ gives consistently higher activity than does ribonuclease A in 3 M ammonium sulfate.

3. When incubated in 3 M ammonium sulfate with substrate, S-protein loses the ability to interact rapidly with S-peptide and to give active ribonuclease S′. The latter finding implies that 3 M ammonium sulfate causes a further structural alteration in S-protein which is prevented when S-peptide is present.

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HISTOLOGICAL STUDIES ON ABSORPTION OF VARIOUS WATER SOLUBLE SUBSTANCES FROM THE PERICARDIAL CAVITY INTO BLOOD VESSELS

Nagoya Med.

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INTRODUCTION

It has been confirmed by numerous studies in the past that various substances introduced into the pericardial cavity are absorbed either by the lymph or blood veseels. However, depending on the nature of the substance introduced the paths of absorption differ, and large particulate substances such as chinese ink[1)-4)], graphite particles[5)] and trypan blue[3)5)] are absorbed directly *via* the lymph vessels of the epi- and pericardium, or after phagocytosis by mononuclear phagocytes in the pericardial cavity[1)5)], are absorbed by lymph vessels, while large proteins such as serum[5)] or large molecular dyes such as vital red[5)] are not demonstrable in blood or lymph vessels. Lithium carmine[7)] can be found in small quantities in the lymph vessels, but carmine[8)-10)] is not absorbed into a lymph or blood vessel. Uranine solution[7)] composed of small molecules and physiological salt solution[5)] have been reported as being absorbed from blood vessels.

Recently, Kotani[1)] reported in detail the path of absorption of chinese ink *via* the lymph vessels of the pericardium. According to him, the mesothelial cells of the serous membrane where absorption occurs are smaller in size when compared with those where absorption does not take place and they are found to exist in groups. Below them the so-called macra cribriformis[11)] of Kihara is present, which has a cribriform structure composed of collagenous fibers and argyrophil fibers. And absorptoin occurs by way of the holes of these networks and through the intercellular space between the endothelial cells of lymph vessels that lie below them. The above view is based on the theory of "extravascular fluid path system" of

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Kihara and his school, which asserts that formed particles such as chinese ink, when found outside a lymph or blood vessel, flow through a special pathway that is related to argylophil fibers.

On one hand, when aqueous solutions of dyes, chemicals and fluorescent substances were used absorption *via* blood vessels was observed by the state of staining with dyes merely macroscopically, or by the appearance of their solutions in the urine. But no studies have been made so far to determine histologically the path of absorption into a blood *via* which particular site and structure. Employing solutions of sodium rhodizonate, barium chloride, ferric citrate, potassium ferrocyanide, ascorbic acid and silver nitrate glacial acetic acid in combination in order to produce coloured precipitation reactions, and also a solution of radioisotope we attempted an investigation histologically of these pathways of absorption.

Materials and Methods

Thirty one puppies, 15 cats, 12 rabbits (body weight 1.5–2.0 kg.) and 3 rats were examined. As substances to be absorbed, those precipitable were selected to be of a size discernible microscopically, well discriminated from the surrounding tissues, and not to dissolve and be lost during the preparation of the specimens. The substances selected were as below, and they were prepared to be as close as possible physiologically as regards osmotic pressure and pH. Also suitable reagents were selected to produce the precipitates of these substances.

Combination→ Type↓	Substances absorbed	Test reagents or fixative reagents
No. 1	0.5% sodium rhodizonate in physiological saline	5% aqueous solution of barium chloride
No. 2	1% ferric citrate in physiological saline	2% potassium ferrocyanide+1% hydrochloric acid
No. 3	2% potassium ferrocyanide in physiological saline	1% ferric citrate+1% hydrochloric acid
No. 4	ascorbic acid (20–40 mg.) in physiological saline	100 ml. of 5% aqueous silver nitrate+5 ml. glacial acetic acid
No. 5	0.9 cc. of $Na_2S^{35}O_4$ in physiological saline	5% barium chloride in 10% formalin

The above aqueous solutions of the substances to be absorbed were introduced slowly into the pericardial cavity in amounts of 0.2–3.0 cc. accord-

ing to size of animal, and after definite intervals of 5, 10, 15, 20, 30 and 40 minutes the animals were sacrificed and the reagents to precipitate the above were introduced slowly *via* the abdominal aorta, in amounts of 50–150 cc. depending on size of animal. Or, the pericardial cavity was opened and the heart with the pericardium was placed in the reagent for 10–20 minutes, and parts of the heart and pericardium where the absorbable substances have been precipitated along the pathway of absorption were removed, fixed in formalin or alcohol, embedded in paraffin or celloidin, and sectioned. In case of $Na_2S^{35}O_4$ the autoradiographs were prepared by the inverting method. Specimens were stained with paracarmine, hematoxylin-eosin, aniline blue, Van Gieson, Bielshowsky-Maresch and Weigert's stains, or examined unstained, or stretch preparations prepared, and the pathways of absorption were observed morphologically.

Results

A. Macroscopic observation

The chemical reactions between sodium rhodizonate and barium chloride, and between ferric citrate and potassium ferrocyanide produced in case of the former a reddish brown precipitate of barium rhodizonate around the site of absorption, and in case of the latter a blue precipitate of berlin blue. In case of ascorbic acid and silver nitrate acetic acid there resulted a black or blackish brown precipitate, while radioisotope and barium chloride produced no reaction recognizable by the naked eye.

Immediately after injection, the reddish brown, blue or blackish brown coloration was seen extremely faintly over the surface of the pericardium, or practically not at all. In other words the state represented merely a mixing of the injected solution with the serous fluid of the pericardial cavity. Five minutes after injection, however, there were seen precipitation and coloration of the sterno-costal surface of the pericardium where the solution was injected, in the form of spots or arborization running along the blood vessels. Coloration of the diaphragmatic and pulmonal surfaces of the epicardium was faint or not recognized.

After 10-20 minutes the surface of the parietal layer (pericardium) rich in adipose tissue became strongly stained with the passage of time, while in other parts the network of blood capillaries became visible. In the visceral layer (epicardium) the blood vessels running along the coronary

groove, and anterior and posterior interventricular grooves became stained and discerned with ease, spreading in the form of an arborization from the base of the heart to the apex. There were also seen spotted areas of staining. The epicardium of the base was stained darkly but of the apex lightly, while that of the auricle was stained relatively faintly in many cases. Areas most darkly stained were parts of the pericardium rich in fatty tissue, the epicardium of the base, epicardial portions around the coronary groove, and anterior and posterior interventricular grooves. Areas stained relatively faintly were parts of the epicardium lacking in fatty tissue and the epicardium of the auricle. The epicardium of the apex was mostly poorly stained. Next a comparison was made of the states of staining of the epicardium and pericardium. It was noted in many cases that the pericardium is stained relatively more darkly, and after 20 minutes staining became gradually more marked, with the blood vessels around the coronary sulcus and anterior and posterior interventricular grooves powerfully stained, and shown up as a dendritic or spotted form. Parts rich in adipose tissue were stained darkly, and the pulmonal and diaphragmatic surfaces, needless to say the sterno-costal surface of the epicardium, were also stained relatively darkly.

After 30–40 minutes the epicardium and pericardium were stained most powerfully, with not only the parts of the pericardium rich in fatty tissue, and in other areas of the pericardium the network of blood capillaries was also clearly stained darkly so that in some parts the contours of blood vessels could not be discerned. The epicardium of the base was also stained very darkly, so also the epicardium of the coronary groove and anterior and posterior interventricular grooves. The epicardium of the apex was relatively faintly stained but the vessels were relatively powerfully stained and presented a dendritic or spotted form. The epicardium of the auricle was also stained darkly throughout. Thus after 30–40 minutes the epicardium and pericardium did not show much difference in grade of staining, and the diaphragmatic, pulmonal and sterno-costal surfaces became stained all over very darkly.

Next, a comparison was made of the state of staining by these water soluble substances. It was then found that ascorbic acid was shown up most clearly as black or blackish brown precipitates, and even after 10–20 minutes the entire area was stained so darkly as to conceal the contours of the blood vessels. Staining with berlin blue appeared after 30–40

minutes, as darkly as with ascorbic acid, while barium rhodizonate produced a reddish brown coloration so that the vessels could not be discerened.

B. Histological findings

Pericardium

The pericardium is covered by a single layer of flat mesothelium. When this is examined as a stretch preparation there is observed 5 minutes after injection of ascorbic acid a widespread dark precipitation reaction, evidenced by invasion into the intercellular spaces of the mesothelial cells (Fig. 1). Compared with the above, barium rhodizonate and berlin blue showed at first only slight precipitation, but after 20-30 minutes precipitates gradually increased in amount to present a state similar as with ascorbic acid, and by filling the intercellular spaces of the mesothelial cells the contours of these cells came to be well shown up (Fig. 2). In the pericardium itself the precipitated granules of ascorbic acid, barium rhodizonate and berlin blue appeared in the intercellular spaces of the mesothelial cells, and also diffusely in the space between fibers of the connective tissues immediately below (Fig. 3). When examined in stretch preparations, collagenous fibers were thick and the spaces between them were narrow in parts where fibers were found to aggregate densely and to run regularly in parallel, and argyrophil fibers could not practically be seen. In parts where the collagenous fibers crossed irregularly the fibers were thin, the spaces between fibers wide and argyrophil fibers were relatively well developed in the interspaces (the macra cribriformis of Kihara). The water soluble substances were found of course to enter in large amounts the wide interspaces, but irrespective of the state of collagenous fibers, whether dense or loose, and the distribution of argyrophil fibers, they were seen to invade diffusely all spaces between fibers. These water soluble substances that have entered the intercellular spaces of the mesothelial cells pass through the spaces of the connective tissue into blood capillaries and venules (Figs. 5, 6). In the artery the surrounding connective tissue showed marked precipitation but not in the walls of arteries. Against this, in blood capillaries and small veins (up to 80 μ), precipitates were seen not only in the lumen, but in between the endothelial cells of the wall, so that the narrow long rhombic shape of the endothelial cells was shown up clearly (Fig. 6), and in parts of pericardium with fatty tissues rich in blood capillaries and blood vessels the precipitates were still more markedly seen in the connective and fatty

tissues. Numerous precipitates were also seen in the blood capillaries and venules of these areas (Fig. 7), corresponding to the macroscopic observations. In the pericardium without fatty tissue the sterno-costal surface was stained strongly after 5–10 minutes and the diaphragmatic and pulmonary surfaces weakly. But after 20–30 minutes the sterno-costal, diaphragmatic and pulmonal surfaces came to show no difference between each other, and to be stained uniformly, strongly and diffusely.

When radioisotope-S^{35} was employed, in stretch preparations black silver grains were seen abundantly all over the pericardium 10 minutes after injection, and there were cases where the figures of the blood capillaries and venules were shown up clearly due to absorption into these vessels (Fig. 8). Again in parts rich in fatty tissue, namely those with abundant blood capillaries and vennles, the black silver grains were seen extremely abundantly (Fig. 9). When S^{35} was employed it is not possible to trace in detail the structure histologically, but in general the picture of absorption corresponded to that of water soluble substances.

Epicardium

As in the case of the pericardium, the epicardium, after 5–10 minutes, revealed in the intercellular substances of mesothelial cells precipitates of barium rhodizonate and berlin blue, as also in the space between the connective tissue fibers (Fig. 10). In the epicardium, the coronary groove and anterior and posterior interventricular grooves are rich in fatty tissue, and also in capillaries and venules, so that compared with other areas of the epicardium there were seen numerous precipitated granules (Fig. 11). They were also seen in between endothelial cells of the blood capillaries and venules, helping to bring out clearly the contour of the endothelial cells. Precipitates were also seen markedly in the tissues around arteries, but not to enter the vascular walls. At the end of 5 minutes the sterno-costal surface of the epicardium near the site of injection showed numerous precipitates, but not in other areas. However at the end of 10 minutes the pulmonal surface of the epicardium and the diaphragmatic surface of the epicardium came also to show numerous deposits. After 20–40 minutes, with passage of time, precipitates came to be seen abundantly throughout the entire epicardium (Fig. 12). In general, more precipitates were seen in the epicardium of the base than of the apex of the heart. The precipitated granules were seen not only in the epicardium but also in between

the muscle fibers of the outer layers of the myocardium (Fig. 13), and to collect around the blood vessels and capillaries, and also in the lumen of capillaries and venules. Here too, precipitates were seen in the tissues surrounding the arterioles, but not in their walls. Precipitates were also seen in the epicardium of the auricles, extending in between the spaces of the loose connective tissues and adipose tissue to the outer layer of the myocardium. In the epicardium of the apex precipitates were found most scarcely at parts lacking fatty tissue, but here too, they were seen to extend to the outer layer of the myocardium, though restricted only to the outermost layer. After 20-40 minutes precipitates of berlin blue and barium rhodizonate were found in the endocardium. This is believed to be due to the invasion of water soluble substances which had entered *via* the veins of the atrium and ventricle to reach the endothelium and subendothelial loose connective tissues of the endocardium. When the epicardium and pericardium were compared more precipitates were found in the pericardium. Next, when S^{35} was employed there were seen numerous silver grains in the epicardium even after 10 minutes, which extended also to the outer layers of the myocardium (Fig. 14, 15). These grains were seen most numerously in the coronary groove and anterior and posterior interventricular grooves, and least in the apical epicardium. When the grades of absorption of the epi- and pericardium were compared, there was seen as in case of water soluble substances a relatively greater absorption in the pericardium (Fig. 15).

Considerations

Embryologically, the pericardial cavity is developed as a serous cavity, the same as the peritoneal and pleural cavities. Hence its cavity is structurally similar to the other two and consists of serous membrane. Of the substances introduced into its various cavities granules with shape and colloidal substances are believed to pass through special parts, namely so-called macra cribriformis, and absorbed *via* the lymph vessels, while small molecular water soluble substances are absorbed *via* the blood vessels.

As was made clear from the results of the present series of investigations, water soluble substances, whether ascorbic acid or sodium rhodizonate, ferric citrate or $Na_2S^{35}O_4$ were found to be absorbed *via* the intercellular spaces of the endothelial cells of blood capillaries and small

veins. Ito[12] studied the absorption of water soluble substances from the pleural cavity, while Yamada[13] that of isotope S^{35} from the peritoneal cavity, and both found that, regardless of the chemical property of the substances to be absorbed, the substances invade diffusely in between the mesothelial cells of the serous membrane, to reach the subserosa after passing the lamina propria, and absorbed from the blood vessels. In the pericardial cavity aqueous solutions also assume a similar attitude and absorption occurs from blood vessels after passing through the intercellular spaces of the mesothelial cells of the serous membrane and extend diffusely in between the collagenous fiber bundles or through the meshes of its network. In this case absorption occurs from all areas of the serous membrane, and not merely from the small type of mesothelial cells of the serous membrane as in the case of absorption of granules such as chinese ink. Also in case of absorption of aqueous solutions the macra cribriformis in contact with the mesothelial cells of the serous membrane seem to play no intimate role. No special relation was also seen with the sorts of fiber, namely collagenous, argyrophil or elastic fibers. Based on the above, small molecular substances may be said to infiltrate diffusely and pass through the serous membrane.

In case of the pleural and peritoneal cavities absorption of aqueous solutions differed according to the locality of the peritoneum or pleura, and a similar phenomenon was also seen in absorption from the pericardial cavity. This was evidenced by the greater absorption from the pericardium than the epicardium, especially when there existed below adipose tissue with good development of blood vessels. In the epicardium too, absorption was especially good at the base of heart, coronary groove, and anterior and posterior interventricular grooves, while absorption from the apex was poor. These findings indicate that absorption occurs with ease in the lamina propria serosae or tela subserosa, where blood vessels are well developed or the interspaces between connective tissue fibers are numerous, namely at places rich in fatty tissue or loose connective tissue.

Summary

Aqueous solutions of sodium rhodizonate, ferric citrate, ascorbic acid and $Na_2S^{35}O_4$ were injected into the pericardial cavity of dogs, cats, rabbits and rats, and a study was made of the pathways of absorption of these

water soluble substances. Results obtained were as follows.

(1) These substances pass between the mesothelial cells of the serous membrane of the pericardium and epicardium, through the connective tissue fibers, between the endothelial cells of the blood capillaries and small veins and are absorbed into the lumen of blood vessels.

(2) In case of water soluble substances, absorption occurs uniformly from all areas of the mesothelium, uninfluenced by the size of the mesothelial cells of the serous membrane, or as in the case of chinese ink from special sites, namely the so-called macra cribriformis of Kihara.

(3) Absorption of water soluble substances was not especially connected with the reticular fibers or other fibrous constituents.

(4) Absorption was at its maximum 20–30 minutes after injection.

(5) Absorption was most marked in parts of the pericardium containing fatty tissue, followed by the epicardium at the base of the heart, the coronary groove and anterior and posterior interventricular grooves. Absoption was poorest at the apex.

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Explanation of Figures

Fig. 1. 5 minutes after injection of ascorbic acid in physiological saline into the pericardial cavity of a dog. Invasion of the solution is seen in between the mesothelial cells of the pericardium. Stretch preparation. Unstained (×280).

Fig. 2. 20 minutes after injection of 0.5% sodium rhodizonate in physiological saline into a rabbit. Invasion of the solution is seen in between the meso-

thelial cells of the pericardium. Stretch preparation. Unstained (×250).

FIG. 3. 30 minutes after injection of 1% ferric citrate in physiological saline into a dog. Invasion of the solution seen in between the connective tissue fibers of the pericardium (pars diaphragmatica). Paracarmine stain (×50).

FIG. 4. Macra cribriformis of the pericardium of a rabbit. 10 minutes after injection of 1% ferric citrate in physiological saline. Stretch preparation. Bielshowsky-Maresh stain (×320).

FIG. 5. Picture of absorption in blood capillaries and small veins of the pericardium of a dog. 5 minutes after injection of ascorbic acid in physiological saline. Stretch preparation. H. E. stain (×70).

FIG. 6. Picture of absorption by a small vein (caliber 80 μ) in the pericardium of a rabbit. 30 minutes after injection of 0.5% sodium rhodizonate in physiological saline. Precipitates are seen in between endothelial cells of the vein and contour of endothelial cells clearly shown up. Stretch preparation. Unstained (×400).

FIG. 7. Pericardium rich in adipose tissue (rabbit). 20 minutes after injection of 2% potassium ferrocyanide in physiological saline. P.C.: pericardial cavity. Van Gieson stain (×70).

FIG. 8. Picture of absorption of S^{35} by blood capillaries and small veins in the pericardium of a rabbit. 10 minutes after injection of $Na_2S^{35}O_4$ in physiological saline. Stretch preparation. Unstained (×70).

FIG. 9. Picture of absorption of S^{35} by blood capillaries and small veins in the pericardium (P) rich in fatty tissue of a rabbit. E: Epicardium. P.C.: Pericardial cavity. H. E. stain (×70).

FIG. 10. Picture of invasion of potassium ferrocyanide into the interspaces of connective tissue fibers of the epicardium of a dog. 5 minutes after injection of 2% potassium ferrocyanide in physiologicai saline. Van Gieson stain (×80).

FIG. 11. Picture of invasion of ferric citrate into the epicardium (E) near sulcus coronarius of a dog. 10 minutes after injection of 1% ferric citrate in physiological saline. Invasion into the pericardium is indicated by P. Unstained (×50).

FIG. 12. The epicardium of the heart of a rabbit. 20 minutes after injection of 1% ferric citrate in physiological saline. Ferric citrate invaded the interspaces of connective tissue fibers, in part into the outer layer of myocardium and absorbed by small blood vessels. Van Gieson stain (×100).

FIG. 13. The pericardium of the heart of a rabbit. 40 minutes after injection of 1% ferric citrate in physiological saline. Ferric citrate invaded the interspaces of muscle fibers of the outer layer of myocardium. Absorption by blood vessels is seen. Van Gieson stain (×100).

FIG. 14. Picture of absorption of S^{35} in the epicardium of the heart of a rabbit. 10 minutes after injection of $Na_2S^{35}O_4$ in physiological saline. Unstained (×80).

FIG. 15. The epicardium (E) and pericardium (P) of the heart of a rabbit. 30 minutes after injection of $Na_2S^{35}O_4$ in physiological saline. Absorbed S^{35} is more abundant in the pericardium than in the epicardium. P.C.: pericardial cavity. Unstained (×80).

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Pharmacology of the Capillary Permeability and the Lymph Secretion. (II)

The Effects of Drugs on the Passage of Dyes from Blood to Lymph and on the Lymph Formation.

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Introductory.

The walls of the blood capillaries are very thin and water and crystalloid substances, except proteins and other colloidal substances, can freely pass through. It is often reported, however, that under various physical and chemical actions, the passage of substances which can pass through the capillary wall become facilitated, and that those substances which can not pass through under normal conditions, become able to do so, *i. e.* the capillary permeability is increased.

But the studies on the influence of drugs on the capillary permeability are not yet satisfactory. It is reasonable to suppose that through the increase of the capillary blood pressure or the variation of osmotic pressure of blood, those substances which can pass through the capillary wall are increased; and that whatever the external stimulation may be, capillary dilatation causes the enlargement of the capillary pore and consequently the increase of the capillary permeability. Nevertheless, according to *Krogh*[1]), the increase of the capillary permeability was sometimes not observed in spite of the slight capillary dilatation. He assumed that the diameter of the capillary depends chiefly upon the tonus of the *Rouget* cells, but the permeability depends absolutely upon the condition of the endotherium. If that be really the case, it is of course hasty to conclude that the permeability of the blood capillaries is increased at times when the filtration from the blood become active due to the increase of

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blood pressure or the variation of osmotic pressure.

In the preceding experiments, I [3]) contrived a method referring to the timely collection of the subcutaneous lymph of toads, and observed the condition of lymph flow, and at the same time confirmed the facts that fuchsin and other many colors could freely pass through the capillary wall and that crystal violet, victoria blue etc. were not detected in lymph.

I attempted further to confirm the condition of lymph flow, the variation of the quantity of colors of the former group in the lymph and the presentation of the latter group in lymph during the course of time the object were under the effects of various drugs, and to observe the effects of those drugs on capillary permeability. Moreover I wished to compare these results with the actions of those drugs on the capillary blood pressure and its diameter, for which see the next chapter. I used in these experiments as the color of the former group fuchsin and as the latter crystal violet, for fuchsin and crystal violet were of those colors which were influenced by the blood serum and the body fluid but slightly, and when crystal violet was injected intravenously, the peripheral vessels exhibited a violet color, yet in spite of that the color was not found in the lymph.

My experiments were carried out on toads, because, as was shown by *Ingvar* [2] and *Conklin* [4]), the water movement from blood to lymph in the frog is so rapid that the entire plasma volume is said to pass through the capillary wall fifty times in twenty-four hours, and the fact that the subcutaneous lymph of toads, differing from that collected from ductus thoracicus of mammals, contains many of the substances filtered through the subcutaneous capillary wall. The toad is therefore very well adapted to such experiments.

Experimental Methods.

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The preparation of the animals and the method of collecting the lymph are the same as were explained in the preceding report. After the preparatory arrangement of the lymph collection and the injection, the drug and the dye solution were injected into V. submaxillaris. Just after the injection, a graduated test tube was placed under the canula through which the lymph flowed out, and it was replaced at intervals of twenty minutes. The measurement of the dye concentration in the lymph was similar to that of preceding experiments. As a matter of course I did my best to avoid the loss of blood in the experiment. The most of drugs employed in the experiments were those on which the lymphagogic action and the effect on the capillary permeability have been studied and discussed hitherto; *i. e.* Adrenalin hydrochloride, pilocarpin hydrochloride, secacornin "*Roche*", coffein, diuretin, theocin, histamin hydrochloride, sodium arsenate, sodium chloride, dextrose and sodium sulphate.

Under the same dose of the same drug the experiments were carried out on ten toads, on which the operations were smoothly performed; and the average value was compared with the results of the contrasts. All the drugs in each dose dissolved in 0.1 c.c. of physiological table salt solution, except in cases of sodium chloride, sodium sulphate and dextrose, in which the volume of injection was 2 c.c. per 100 g. of the body weight and the concentration was varied. Comparatively large doses of the drugs were employed in my experiments, for the action of the drug on toad is not so marked as in the case of mammals. But if the dose was too large, the capillary was attacked and blood corpuscles were mixed in lymph, therefore such a dose was not adopted in my experiments. The

contrast is shown in the results obtenaid in experiments in normal toads in which only the dye solution was injectioned.

Experimental Results.

In my experiments, the lymph formation and the passage of dyes from blood to lymph under various drugs are as follows. The results are also shown in tables printed under their descriptions.

***Adrenalin*:** 0.01 mg. of adrenalin hydrochloride per 100 g. of the body weight of toad was injected intravenously, and the flow of lymph was remarkably increased. The volume of lymph flow in two hours was greater than that in the case of the maximum value of the contrasts, to say nothing of the average value of them. But considering the volumes collected over each 20 minutes period, in sixty minutes from the injection of the drug it was far greater than that in the case of the contrast. In spite of that it decreased in comparison with the contrast after that time. The concentration of dye in lymph was increased during the first 20 minutes and reached the maximum in 20 to 40 minutes. Sixty minutes after the injection, it was rather less than that of the contrast. When four times the dose of the drug was injected, the increase of the lymph formation and the concentration of dye in the lymph was very marked.

***Pilocarpin*:** When 0.01 mg. of pilocarpin hydrochloride, per 100 g. of the body weight, was injected, the action of the drug was not marked; when 0.1 mg. of the drug was given, the lymph formation seemed to be increased, but was far inferior to that in the case of adrenalin hydrochloride, and the quantity of dye which appeared in the lymph showed only a slight increase. When 0.2 mg. of the drug was employed, the quantity of dye was not much different from that of the contrast in spite of the decided increase of the lymph formation.

***Secacornin "Roche"*:** When 0.1 c.c. of the drug, per 100 g. of the body weight, was injected subcutaneously, the action was not remarkable. But in the case of an intravenous application, the volume of lymph in two hours showed a slight increase, although the quantity of dye in the lymph was not much different from that of the contrast. Consulting their timely variations, the flow of lymph reached the maximum in the first 20 minutes, in the next 40 minutes it was greater than the contrast and 60 minutes after the injection was less than the contrast. The concentration of dye in the lymph reached its maximum in 20 to 40 minutes after the injection and maintained the moderate value until the end of two hours.

Under these three drugs, crystal violet, which was not detected in lymph under normal conditions, was also not found in lymph after its intravenous injection.

Table 1. The effects of adrenalin hydrochloride, pilocarpin hydrochloride and secacornin "*Bele*" on the passage of dye from blood to lymph and on the lymphformation

Drugs	Injection per 100 g. of the body weight	Time (m.)	0—20	20—40	40—60	60—80	80—100	100—120	Total
Contrast		Vol. of lymph (c.c.)	1.30	1.10	1.02	1.15	0.69	0.32	5.58
		Conc. of dye (%)	0.00013	0.00025	0.00025	0.00023	0.00010	0.00010	
Adrenalin hydrochloride	0.01 mg.	Vol. of lymph (c.c.)	2.63	1.80	1.48	0.78	0.52	0.50	7.51
		Conc. of dye (%)	0.00011	0.00041	0.00038	0.00010	0.00010	0.00010	
Adrenalin hydrochloride	0.04 mg.	Vol. of lymph (c.c.)	2.89	2.21	1.42	0.82	0.77	0.53	8.64
		Conc. of dye (%)	0.00028	0.00037	0.00075	0.00043	0.00017	0.00010	
Pilocarpin hydrochloride	0.1 mg	Vol. of lymph (c.c.)	1.72	1.52	1.31	0.76	0.71	0.30	6.32
		Conc. of dye (%)	0.00010	0.00025	0.00025	0.00025	0.00010	0.00010	
Secacornin "*Bele*"	0.1 c.c. intravenously	Vol. of lymph (c.c.)	2.00	1.70	1.34	0.98	1.00	0.60	7.62
		Conc. of dye (%)	0.00032	0.00054	0.00050	0.00050	0.00050	0.00050	
Secacornin "*Bele*"	0.1 c.c. (hypodermic)	Vol. of lymph (c.c.)	1.70	1.06	1.56	0.48	0.75	0.54	6.09
		Conc. of dye (%)	0.00010	0.00025	0.00025	0.00025	0.00025	0.00025	

The experiments were carried out on ten toads in each dose of the drug, and the average values are shown in the table.

***Coffein*:** When 0.02 g. of coffein, per 100 g. of the body weight, was injected, the volume of lymph had a tendency to slightly increase, but the contrast. By the application of 0.04 g. of the drug, the increase of lymph flow in two hours was remarkable. The concentration of dye in the lymph was greater during the first hours but during the next hour it was a little less than that of the contrast.

***Diuretin and theocin*:** When these drugs were mixed with a solution of fuchsin, the colortone was diluted and faded in a short time. And in these experiments 0.1 g. of the drug, per 100 g. of the body weight, was injected intravenously 10 minutes before the injection of the dye solution. The quantity of dye in the lymph was from the beginning less than in the contrast in spite of the decided increase of the lymph flow. The actions of these drugs can not be briefly concluded from these facts because of the fading effect of these drugs themselves. Under these three drugs, crystal violet, which could not be detected in lymph in normal conditions, was also not found in lymph after its intravenous injection.

Table 2. The effects of coffein, theocin and diuretin on the passage of dye from blood to lymph and on the lymphformation.

Drugs	Injection per 100 g. of the body weight	Time (m.)	0—20	20—40	40—60	60—80	80—100	100—120	Total
Contrast		Vol. of lymph (c.c.)	1.30	1.10	1.03	1.15	0.69	0.32	5.58
		Conc. of dye (%)	0.00013	0.00025	0.00025	0.00023	0.00010	0.00010	—
Coffein	0.02 g.	Vol. of lymph (c.c.)	1.46	1.18	1.18	0.96	0.74	0.62	6.04
		Conc. of dye (%)	0.00018	0.00028	0.00062	0.00010	0.00010	0.00010	
Coffein	0.04 g	Vol. of lymph (c.c.)	1.94	1.34	1.24	0.96	0.90	0.42	6.82
		Conc. of dye (%)	0.00038	0.00032	0.00010	0.00036	0.00025	0.00018	
Theocin	0.1 g	Vol. of lymph (c.c.)	1.84	1.36	0.82	0.86	0.62	0.66	6.16
		Conc. of dye (%)	0.00005	0.00016	0.00005	0.00005	0.00010	0.00010	
Diuretin	0.1 g.	Vol. of lymph (c.c.)	1.21	1.07	1.06	0.79	1.12	0.80	6.08
		Conc. of dye (%)	0.00003	0.00012	0.00005	0.00003	0.00010	0.00010	

The experiments were carried out on ten toads in each dose of the drug, and the average values are shown in the table.

***Histamin and sodium arsenate*:** Histamin hydrochloride, even in a small quantity, increases the subcutaneous lymph of toad, as does sodium arsenate. When 0.2 mg. of histamin or 0.2 mg. of sodium arsenate, per 100 g. of the body weight, was injected intravenously, the flow of lymph was remarkably increased. The flow reached its maximum within 20 minutes after the injection and during next 60 minutes it was larger than that of the contrast, but after that it had a tendency to decrease. The concentration of dye in the lymph was larger than that of the contrast and reached its maximum in 20 to 40 minutes after the injection. The increase of the quantity of dye in lymph continued for a longer time the case of sodium arsenate than in that of histamin. In the case of arsenic, but never in the case of histamin, crystal violet, which could not be detected in lymph under normal conditions, appeared in lymph, but the concentration was too low to determine the real value. When a large dose of arsenic was applied, the lymph was mixed with many blood corpuscles.

Table 3. The effects of histamin hydrochloride and sodium arsenate on the passage of dye from blood to lymph and on the lymphformation.

Drugs	Injection per 100 g. of the body weight	Time (m.)	0—20	20—40	40—60	60—80	80—100	100—120	Total
Contrast		Vol. of lymph (c.c.)	1.30	1.10	1.02	1.15	0.69	0.32	5.58
		Conc. of dye (%)	0.00013	0.00025	0.00025	0.00023	0.00010	0.00010	

Drugs	Injection per 100 g. of the body weight	Time (m.)	0—20	20—40	40—60	60—80	80—100	100—201	Total
Histamin hydrochloride	0,2 mg.	Vol. of lymph (c.c.)	2.50	2.14	0.94	1.14	0.94	0.50	8.16
		Conc. of dye (%)	0.00016	0.00050	0.00025	0.00025	0.00025	0.00025	
Sodium arsenate	0,2 mg.	Vol. of lymph (c.c.)	2.39	2.22	1.28	0.80	0.64	0.51	7.87
		Conc. of dye (%)	0.00010	0.00050	0.00050	0.00050	0.00035	0.00025	

The experiments were carried out on ten toads in each dose of the drug, and the average values are shown in the table.

Lymphagogues of the second class: In my experiment were used isotonic and hypertonic solutions of sodium chloride and dextrose, and hypertonic solution of sodium sulphate, which were thought as to make the blood watery. When 2 c.c. of isotonic solution of sodium chloride or dextrose, per 100 g. of the body weight, was injected, a slight increase in the flow of lymph and the quantity of dye in lymph was observed, but with the same volume of hypertonic solution, the action was considerable. Within 80 minutes the volume of lymph and the concentration of dye in the lymph always exceeded the contrast; and the increase of the lymph flow in two hours decided. In the cases of other drugs the increase of lymph was limited within 40 minutes after the injection, while in these cases the increased lymph flow continued for a long time. Moreover, crystal violet was detected in lymph under hypertonic solutions of these drugs. The action seemed to be more remarkable in the case of dextrose than in the case of sodium chloride.

When 1 c.c. of sodium sulphate, per 100 g. of the body weight, was given, the increase of the lymph flow was remarkable, but the concentration of dye in the lymph was remarkably decreased. The sulphate has a fading action on the dye solution; thus the action of sodium sulphate can not be concluded to be the same as in the case of other drugs.

Table 4. The effects of sodium chloride, dextrose and sodium sulphate on the passage of dye from blood to lymph and on the lymphformation.

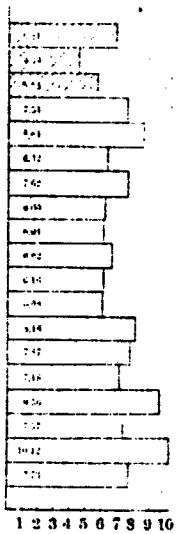
Drugs	Injection per 100 g. of the body weight	Time (m.)	0—20	20—40	40—60	60—80	80—100	100—120	Total
Contrast		Vol. of lymph (c.c.)	1.30	1.10	1.02	1.15	0.69	0.32	5.58
		Conc. of dye (%)	0.00013	0.00025	0.00025	0.00025	0.00010	0.00010	
Sodium chloride	0,6 %	Vol. of lymph (c.c.)	1.96	1.40	0.84	1.16	1.00	0.82	7.18
	2,0 c.c.	Conc. of dye (%)	0.00001	0.00050	0.00030	0.00025	0.00018	0.00010	
Sodium chloride	3,5 %	Vol. of lymph (c.c.)	2.68	2.54	1.36	1.44	0.90	0.64	9.56
	2,0 c.c.	Conc. of dye (%)	0.00027	0.00072	0.00050	0.00010	0.00025	0.00025	

Drugs	Injection per 100 g. of the body weight	Time (m.)	0—20	20—40	40—60	60—80	80—100	100—120	Total
Dextrose	4,0 %	Vol. of lymph (c.c.)	2.48	1.13	0.94	0.50	1.28	0.94	7.57
	2,0 c.c.	Conc. of dye (%)	0.00015	0.00040	0.00050	0.00030	0.00025	0.00013	
Dextrose	20 %	Vol. of lymph (c.c.)	2.70	2.16	1.56	1.46	1.24	1.00	10.12
	2,0 c.c.	Conc. of dye (%)	0.00021	0.00100	0.00037	0.00050	0.00023	0.00023	
Sodium sulphate	5,0 %	Vol. of lymph (c.c.)	1.30	1.64	0.42	1.30	1.00	0.7	7.73
	1,0 c.c.	Conc. of dye (%)	0.00004	0.00001	0.00001	0.00001	0.00001	0.00001	0.00001

The experiments were carried out on ten toads in each dose of the drug, and the average values are shown in the table.

Summary.

The results of my experiments are summarized as follows. Adrenalin hydrochloride, pilocarpin hydrochloride, secacornin, coffein, diuretin, theocin, histamin hydrochloride, sodium arsenate, isotonic and hypertonic solutions of sodium chloride, dextrose and hypertonic solution of sodium sulphate, increase the subcutaneous lymph formation of toads in the course of two hours. Comparing the results obtained with suitable dose of the drug, the action was the most



Contrast (maximum)
" (minimum)
" (average)
Adrenalin hydrochloride per 100 g. 0.01 mg.
" " 0.04 mg.
Pilocarpin hydrochloride " 0.1 mg.
Secacornin "*Ebbs*" " 0.1 c.c.
(intravenously)
" (hypodermic) " 0.1 c.c.
Coffein " 0.02 g.
" " 0.04 g.
Theocin "*Roche*" " 0.1 g.
Diuretin " 0.1 g.
Histamin hydrochloride " 2.0 mg.
Sodium arsenate " 0.2 mg
Sodium chloride (0.6 %) " 2.0 c.c.
" " (3.5 %) " 2.0 c.c.
Dextrose (4.0 %) " 2.0 c.c.
" (20 %) " 2.0 c.c.
Sodium sulphate (5 %) " 1.0 c.c.

Fig. 1. The subcutaneous lymphformation in two hours under various drugs.
The experiments were carried out on ten toads in each dose of the drug, and the average values are shown in the figure.)

remarkable with hypertonic solutions of sodium chloride and dextrose, and secondary with adrenarin, histamin, secacornin and sodium sulphate. When other drugs were used the action cannot be said to be remarkable. The lymph formation with these drugs always larger than the contrast within forty minutes from the beginning, but after that in some cases it was reduced to the contrast or a little below it. With three drugs, contained in the lymphagogues of the second class, the increase of lymph flow continued for a long time, especially in the cases of hypertonic solutions of sodium chloride and dextrose.

The concentration of dye in lymph with all drugs except diuretin, theocin and sodium sulphate which have tendencies to decrease the colortone in the test tube, seemed to increase proportionally with the lymph formation. Considering the lymph volume collected every twenty minutes, the maximum of lymph formation lies in the first twenty minutes and that of the dye concentration in the second twenty minutes. The increase of lymph under secacornin was not remarkable, but the increased dye concentration was maintained for a long time compared to other drugs. The same tendency was observed in the case of arsenic.

The increase of lymph flow and dye concentration under hypertonic solutions of sodium chloride and dextrose, and arsenic, was remarkable. Moreover crystal violet, which was not found in lymph in normal conditions, was detected in this lymph. The increase of the dye concentration was generally accompanied by that of lymph flow, therefore the increase of the absolute quantity of dye, purged into the lymph, was remarkable.

The results of these experiments will be discussed in the next report.

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The Dynamics of Sulfur Dioxide Inhalation

Absorption, Distribution, and Retention

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The combustion of coal and fuel oil has resulted in a considerable contamination of the atmosphere by SO_2, amounting to as much as 2,000 tons per day in a large city such as New York.[1] The effects of the chronic inhalation of low concentrations of this substance are of particular importance, as urban atmospheric concentrations usually do not exceed 0.5 to 1 ppm. This aspect of SO_2 toxicity has been admirably reviewed by Greenwald.[2] The Committee on Threshold Limits of the American Conference of Governmental Industrial Hygienists in April, 1958, recommended that the threshold value for sulfur dioxide be limited to 5 ppm (13 mg. per cubic meter).[3] This is of significance, since the physiological effects of the inhalation of prolonged low-level concentrations of SO_2 in the human are essentially unknown.

Many studies[4,5] have dealt with the effects of relatively short exposures and high concentrations of SO_2, with observation of the symptoms and signs of the accompanying irritation of the respiratory tract. Recently the physiologic effects of exposure to SO_2 have come under investigation.[6-8] In addition, the rapidity of the absorption, distribution, and the length of time sulfur dioxide resides in plants[9] and animals[10] have been investigated.

Both phases of this problem have been of interest to us. A study is in progress in which Sprague-Dawley rats are being exposed to 0, 1, 2, 4, 8, 16, and 32 ppm of SO_2 throughout their lifetimes and for 24 hours each day. Observations include the length of life-span and the resulting histopathological changes. In addition, studies have been completed and are in publication regarding acute exposures of dogs to various concentrations of $S^{35}O_2$, inhalation being via a tracheostomy[11,12] and by nose and mouth breathing.[13] It has been demonstrated in the former study that SO_2 tagged with $S^{35}O_2$ is rapidly absorbed from the trachea and lungs and is distributed to all tissues examined, including the brain. A relatively high per cent retention of the inhaled gas by the trachea, lungs, hilar lymph nodes, liver, spleen, and kidneys occurred. The latter study indicated that when $S^{35}O_2$ is inhaled through the nose and mouth in concentrations of from 1 to 141 ppm a large proportion is deposited in the upper airways, a greatly reduced amount reaching the trachea, lungs, and other viscera. Even so, the smaller quantity of $S^{35}O_2$ reaching the lungs is absorbed and distributed to all tissues, including brain and bone marrow.

The present report is concerned with three additional aspects of interest in regard

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TABLE 1.—*Pulmonary Function in Dogs Exposed to $S^{35}O_2$ only Through a Tracheal Segment in Vivo*

Dog No., Wt.	Length of Tracheal Seg. Exposed, In.	Inhaled Conc. SO_2, ppm	Exhaled Conc. SO_2, ppm	Decrease in Compliance of Lungs & Thorax *		Increase in Pulmonary Resistance †	
				At End of Exp. Period, %	1 Hr. After Exp., %	At End of Exp. Period, %	1 Hr. After Exp., %
1 33 lb.	4½	45	17	12	18	17	20
2 26 lb.	2	91	66	2	3	4.5	0

* In millimeters per centimeter H_2O pressure.
† In centimeters H_2O pressure per liter per second.

to SO_2 transfer. These are (1) the extent of absorption of SO_2 from the trachea when it alone is exposed to SO_2, (2) the length of time that SO_2 can remain in the tissues, and (3) the distribution of $Na_2S^{35}O_4$ when injected into the blood stream, in contrast to the distribution after $S^{35}O_2$ inhalation.

Methods and Results

SO_2 Absorption from Trachea.—Two dogs were prepared so that an isolated segment of the trachea could be perfused with an $S^{35}O_2$ air-gas mixture, without the latter entering the main-stem bronchi or lungs. This was done by opening the chest in the midline through a sternum-splitting procedure and transecting the trachea about 1 in. above its bifurcation. A right-angle metal cannula was inserted into the distal trachea, and the lungs were ventilated only with air. A similar metal cannula was also inserted into the proximal part of the trachea, that is, the end just above the point of transection. This cannula served as the port of entry of $S^{35}O_2$. The chest then was tightly sutured around the metal cannulae and the air remaining in the pleural space aspirated. A tracheal tube with a rubber cuff was inserted through the mouth, and its tip situated just below the larynx. $S^{35}O_2$ gas could thus be pumped from a Mylar bag into the trachea and collected in a second Mylar bag as it exited through the upper tracheal tube. The whole animal was placed in a body plethysmograph so that the compliance of the lungs and thorax and the pulmonary resistance could be measured.[12,13] Pulmonary function measurements were determined serially during a 30-minute control period while the animal breathed air, during an exposure period when $S^{35}O_2$ was pumped through the isolated tracheal segment at a constant rate, and finally during a one-hour postexposure period when the tracheal segment was again perfused with air. The lungs were ventilated with air at all times by a respiration pump.

There was an appreciable decrease in compliance in the first dog and less decrease in the second animal (Table 1). Both demonstrated an increase in pulmonary resistance. These changes in compliance are of the same order of magnitude as seen in animals having their trachea and lungs directly exposed to SO_2.[11,12] The increases in pulmonary resistance are significant in degree and never have been observed to occur spontaneously. They may be com-

TABLE 2.—*Per Cent Retention by Organs After Passage of $S^{35}O_2$ Through an Isolated Segment of Dog Trachea in Vivo*

Dog No.	Vol. $S^{35}O_2$, L.	Exp. Period, Min.	Total Activity S^{35}, μc.	Visceral % Retention of S^{35}				
				Trachea	Lungs	Liver	Spleen	Kidneys
1 *	100	16	72	2.2	1	2.2	.2	.6
2 †	300	35	150	.6	.3	.5	.05	.1

* Length of trachea exposed, 4½ in.
† Length of trachea exposed, 2 in.

TABLE 3.—*Concentrations of S^{35} in Tissues After Isolated Tracheal Segment Perfusion by $S^{35}O_2$ in Vivo* *

	Dog 1	Dog 2
Trachea	260	170
Lungs	5.2	3.6
Hilar lymph nodes	6.2	4.0
Liver	2.7	2.7
Spleen	3.9	2.0
Kidneys	4.9	3.0
Esophagus	5.0	8.4
Stomach	3.5	2.0
Duodenum	2.6	2.0
Jejunum	2.8	2.6
End-exposure, venous blood	2.6	3.9
End-exposure, arterial blood	8.4	4.7

* In millimicrocuries per gram.

pared to an average increase in pulmonary resistance of 230% as seen in animals whose lungs are directly exposed to SO_2[11,12] and 47% in animals breathing SO_2 through the nose and mouth.[13]

$S^{35}O_2$ was readily absorbed from these limited lengths of trachea (Table 2), and an appreciable amount of S^{35} was detected in the lungs, liver, spleen, and kidneys. Tissue S^{35} (Table 3) determinations indicated that concentrations were greater in the lungs, hilar lymph nodes, and midesophagus than in the remaining tissues assayed. The tracheal concentration was, of course, many times that in any other tissue.

Length of Time S^{35} Remains in Tissues.

Two dogs were exposed to $S^{35}O_2$ in the body plethysmograph via a tracheal tube inserted through the mouth.[12] Definite decreases in compliance (Table 4) and increases in pulmonary resistance occurred. They were then returned to their cages and autopsied two and seven days after exposure, respectively, and their tissues analyzed for S^{35} (Table 5). Ninety per cent or more of the $S^{35}O_2$ per unit of gas-air mixture inhaled was retained (Table 4). Even at the end of seven days appreciable concentrations of S^{35} were present in the tissues of the respiratory tract when compared to two animals acutely exposed to similar total amounts of S^{35} via a tracheostomy (Dogs 8 and 12, Table 5). The S^{35} concentration in the other viscera and tissues was either very low or nil. Therefore, the trachea and lungs retain S^{35} for as long as seven days after acute exposures to moderate concentrations of $S^{35}O_2$.

No S^{35} was detected in the feces at any time, but the S^{35} concentrations in the urine were 11.7 mμc. per milliliter in Dog 3 two days after exposure and 0.7 mμc. per milliliter in Dog 4 seven days after exposure.

Distribution of Intra-Arterially Injected $Na_2S^{35}O_4$.—One thousand microcuries of S^{35} in the form of $Na_2S^{35}O_4$ was injected into the femoral artery of a dog. Blood samples were taken for 100 minutes, and the dog was then autopsied. This time corresponds to that used in dogs which have inhaled $S^{35}O_2$ (40-minute exposure period plus a 60-minute postexposure follow-up period).[11-13] The concentrations in the tissues and per cent retentions by the organs were very low. Only 0.06% was retained by the trachea and 0.2% by the lungs. The

TABLE 4.—*Lung Function Tests During Exposure to $S^{35}O_2$-Tagged SO_2* *

Dog No., Wt.	SO_2 Inhaled, ppm / μg/100 Cc.	SO_2 Exhaled, ppm / μg/100 Cc.	SO_2 Retained, μg. / μg/lb.	Total Exp. Time, Min.	Compliance of Lungs & Thorax † Control	Lowest	Final	60 Min. After Exp.	Pulmonary Resistance ‡ Control	Lowest	Final	60 Min. After Exp.	Autopsy
3 27 lb.	15 / 3.93	1.2 / 0.37	3024.1 / 112	42.5	19.4	19.6	19.6	18.8 −3.1%	54.0	66.5 +23%	66.5	54.5	2 days after SO_2 exp.
4 27 lb.	33 / 8.64	1.3 / 0.35	6615.4 / 245	40.0	22.6	22.0	21.8	21.6 −4.4%	27.4	55.0 +101%	48.0	28.7	7 days after SO_2 exp.

* Per Cent figures are increases and decreases, compared to control values.
† In millimeters per centimeter H_2O pressure.
‡ In centimeters H_2O pressure per liter per second.

TABLE 5.—S^{35} *Retained in Tissues and Organs*

	Dog 3, Autopsied After 2 Days		Dog 4, Autopsied After 7 Days		Acute Tracheal Exposure % S^{35} Retained *	
	S^{35}, Mμc/Gm.	% S^{35} Retained	S^{35}, Mμc/Gm.	% S^{35} Retained	Dog 12	Dog 8
Weight, lb.	27		27		43	28.5
Exp. time, min.	42.5		40		20	35.0
ppm	15		33		11.2	29.8
Total S^{35} retained, μc.	21.3		33.7		14.5	36.9
Larynx	—	—	1.4	0.07	—	—
Trachea	15	0.6	1.8	0.9	7.2	6.0
Lungs	0.4	0.23	0.42	0.11	6.9	2.4
Hilar lymph nodes	0	0	0	0	0.2	0.003
Abdom. lymph nodes	0	0	3.5	0.06	—	—
Right vent. muscle	0	0	0	0	0.6	—
Left vent. muscle	0	0	0	0	2.3	0.5
Liver	0	0	0	0	27.4	10.2
Spleen	0	0	0	0	5.4	1.0
Bile	0.7	—	0	0	—	—
Kidneys	0	0	7.8	0.02	2.8	1.9
Urin. bladder	0	0	0	0	—	—
Adrenals	0	0	0	0	—	—
Pancreas	0	0	0	0	—	—
Testes	0.2	0.02	0	0.01	—	—
Thyroid	2.4	0.14	1	0	—	—
Submax. gland	0	0	0	—	—	—
Esophagus	4.6	0.9	—	—	—	—
Fundus stomach	0.6	—	1.5	—	—	—
Jejunum	0.5	—	0.7	—	—	—
Brain	—	—	0	0	—	—
Skin	0	0	0	0	0.6	—
Muscle	0	0	0	0	—	—
Eye	0	0	0	0	—	—
Tongue	0.03	—	—	—	—	—
	0.36	—	0.1	—	—	—

* Two dogs exposed via tracheostomy and autopsied 40 minutes after end of inhalation period. Figures given are in per cent of S^{35} which was retained by animal which is present in organ or tissue.

liver, spleen, kidneys, and brain retained 4.6%, 0.26%, 0.3%, and 0.6%, respectively, of the administered radioactive sulfate. The concentrations in the trachea and lungs were much lower than those in animals inhaling $S^{35}O_2$ in amounts which were only 1/10 of the activity which had been given intra-arterially. The remaining tissue concentrations were similar to those found in dogs receiving $S^{35}O_2$ by inhalation.[12,13] Therefore, S^{35} reaching the blood stream either after inhalation of $S^{35}O_2$ or by injection as $Na_2S^{35}O_4$ is distributed and becomes localized in a similar pattern in tissues other than those of the respiratory tract. No localization in the tissues of the respiratory tract follows intravenous administration of radioactive sulfate.

Comment

When inhaled through the nose and mouth in concentrations as low as 1 ppm, SO_2 is absorbed and reaches all tissues and viscera, including the brain.[11-13] The majority of the SO_2 (90% or more) is removed from an inhaled gas-air mixture and becomes localized mainly in the pharynx, trachea, lungs, and hilar lymph nodes. It is taken up from these sites of deposit, and a significant portion is found in the liver, spleen, and kidneys.

The SO_2 in the trachea and lungs is only slowly removed from these sites and, according to our experiments, can remain in these tissues for at least one week. According to Bystrova,[10] radioactive sulfur may be found in the tissues of mice, especially in the lungs, for as long as three weeks after exposure. It was detected in the protein portions of the tissues.

S^{35} is readily excreted in the urine, but none was found in the feces. It was stated to be excreted in both feces and urine

by Bystrova,[10] but in these experiments high concentrations of SO_2 were used and also a "priming dose" technique was used.

It was demonstrated by our tracheal transpiration experiments that the tracheal mucosa, when alone exposed, can absorb SO_2 in sufficient amounts to affect pulmonary function. Similar tests were not carried out to test uptake by the mucosa of the nose and mouth. However, $S^{35}O_2$ becomes deposited in these absorptive sites in high concentrations after nose and mouth breathing of this gas, and uptake into the blood most likely follows.[13]

The chemical nature of SO_2 as it exists in the tissues and blood after absorption has been little studied. Bystrova[10] has stated that SO_2 is attached in some manner to the proteins of the plasma and tissues. The mechanism by which pulmonary resistance and compliance are affected by SO_2 absorbed into the blood after tracheal exposure alone is unknown. Reflex bronchoconstriction after stimulation of nerve endings in the bronchi is possible, but a direct effect upon smooth muscle also has to be considered. In our preparation the nerve supply to the isolated tracheal segment as well as the bronchi and lungs was not disturbed.

S^{35}, given as $Na_2S^{35}O_4$ intra-arterially, did not become preferentially localized in the trachea and lungs and apparently became uniformly distributed throughout the body water.

Summary

SO_2 is readily absorbed from the upper and lower respiratory tract even when inhaled in low concentrations (1 ppm). It can be asbsorbed by the tracheal mucosa when the latter alone is exposed to a degree sufficient detrimentally to influence pulmonary function. After absorption from the sites of deposition in the trachea, bronchi, and lungs, it is distributed to all tissues, including the brain. It can still be detected in the trachea and lungs one week after exposure. The physiologic and metabolic effects of the prolonged inhalations of low-level concentrations of SO_2 are unknown, and experiments are now in progress in an attempt to obtain further data on this aspect of the effects of the inhalation of low concentrations of sulfur dioxide.

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2.3-Diphospho-glycerat-Stoffwechsel und Glykolyse in Menschen-Erythrozyten
Einfluß von Sulfat, Tetrathionat und Disulfit

Von JOCHEN DUHM, BERNHARD DEUTICKE und ECKEHART GERLACH

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Zusammenfassung: Der spontane Abfall der 2.3-Diphospho-glycerat-Konzentration in Menschen-Erythrozyten bei Inkubation im eigenen Plasma (pH 7,35; 20mM Glucose; 37°C) wird durch Dithionit, Sulfit und Disulfit (5mM) stark beschleunigt, durch Sulfat, Thiosulfat und Tetrathionat (1—5mM) gehemmt bzw. verhindert. Diese Effekte sind auch in Glucose-frei inkubierten Hämolysaten bzw. Erythrozyten, in denen keine 2.3-Diphospho-glycerat-Synthese stattfindet, nachweisbar, und beruhen auf einer direkten Aktivierung bzw. Hemmung der Diphosphoglycerat-Phosphatase.

Der Glucose-Verbrauch des Erythrozyten wird durch alle untersuchten Schwefel-Verbindungen um 50—100%, die Lactat-Bildung um 20—80% gesteigert. Dabei nehmen die Konzentrationen von Fructose-1.6-diphosphat, Dihydroxyacetonphosphat und Glycerinaldehyd-3-phosphat zum Teil erheblich zu. Die Stimulation der Glykolyse dürfte in erster Linie durch Aktivierung der Phosphofructokinase verursacht sein.

Summary: *Metabolism of 2,3-diphosphoglycerate and glycolysis in human erythrocytes. The influence of sulphate, tetrathionate and disulphite.* The spontaneous decrease of 2,3-diphosphoglycerate in human erythrocytes incubated in plasma (pH 7,35; 20mM glucose; 37°C) is considerably enhanced by dithionite, sulphite and disulphite (5mM), and is strongly retarded or even completely inhibited by sulphate, thiosulphate and tetrathionate (1—5mM). These changes could also be registered in erythrocytes incubated in glucose-free media and in hemolysates where the disappearance of 2,3-diphosphoglycerate is accelerated due to the lack of synthesis. Thus it can be concluded that the effects of the sulphur compounds result mainly from a direct activation or inhibition of diphosphoglycerate phosphatase, respectively.

All sulphur compounds studied increase glucose consumption of erythrocytes by 50—100%, and

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Enzyme:

Diphosphoglycerat-Phosphatase, 2.3-Diphospho-D-glycerat-2-Phosphohydrolase (EC 3.1.3.13)
Glycerinaldehydphosphat-Dehydrogenase, D-Glycerinaldehyd-3-phosphat:NADP-Oxydoreduktase (EC 1.2.1.9)
Phosphofructokinase, ATP:D-Fructose-6-phosphat-1-Phosphotransferase (EC 2.7.1.11)
Phosphoglyceromutase, 2.3-Diphospho-D-glycerat:2-Phospho-D-glycerat-Phosphotransferase (EC 2.7.5.3)
Phosphopyruvat-Hydratase, 2-Phospho-D-glycerat-Hydro-Lyase (EC 4.2.1.11)
Pyruvat-Kinase, ATP:Pyruvat-Phosphotransferase (EC 2.7.1.40)

lactate production by 20—80%. Concomitantly the concentrations of fructose 1,6-diphosphate, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate increase considerably but to a varying extent. The stimulation of the glycolysis is assumed to be primarily due to an activation of phosphofructokinase comparable to that induced by orthophosphate.
The results are discussed with respect to possible mechanisms of action of the different sulphur compounds on 2,3-diphosphoglycerate metabolism and on the glycolysis of erythrocytes.

Die hohe 2.3-Diphospho-glycerat-Konzentration in menschlichen Erythrozyten hat eine wesentliche funktionelle Bedeutung: Neueren Befunden verschiedener Autoren zufolge vermindert 2.3-Diphospho-glycerat die Sauerstoff-Affinität des Hämoglobins und begünstigt dadurch die Sauerstoff-Abgabe im Gewebe[1].
Damit gewinnt die Frage, wie die 2.3-Diphospho-glycerat-Konzentration in menschlichen Erythrozyten reguliert wird, an Interesse. In letzter Zeit konnte bewiesen werden, daß die Geschwindigkeit der Synthese von der ADP-Konzentration abhängt[2]. Bildung und Abbau von 2.3-Diphospho-glycerat stellen unter physiologischen Bedingungen einen Nebenweg der Glykolyse dar, der nur zu 15—20% am Abbau der Glucose beteiligt ist[2,3].
Der 2.3-Diphospho-glycerat-Umsatz läßt sich durch verschiedene Substanzen in spezifischer Weise beeinflussen: Nach Resultaten von MÁNIAY und VÁRADY induzieren die Schwefel-Verbindungen Dithionit und Sulfit in intakten Menschen-Erythrozyten und Hämolysaten einen schnellen Abbau von 2.3-Diphospho-glycerat[4]. Eigenen Befunden zufolge wird der bei mehrstündiger Inkubation von Erythrozyten unter physiologischen Bedingungen auftretende spontane Konzentrationsabfall von 2.3-Diphospho-glycerat durch Dipyridamol und andere Pyrimidopyrimidin-Verbindungen gehemmt[2,5].
Zur Klärung der Frage, welche Mechanismen der Wirkung von Dithionit und Sulfit auf den 2.3-Diphospho-glycerat-Abbau zugrunde liegen[4,6], analysierten wir den Einfluß dieser und anderer anorganischer Schwefel-Verbindungen auf den 2.3-Diphospho-glycerat-Stoffwechsel und die Glykolyse im Menschen-Erythrozyten. Als wesentliches Resultat ergab sich dabei, daß einige der geprüften Substanzen den 2.3-Diphospho-glycerat-Abbau hemmen, und daß alle Verbindungen die Glykolyserate des Menschen-Erythrozyten beträchtlich erhöhen.

Methodik

Versuche an intakten Erythrozyten

Frisch entnommenes menschliches Blut wurde mit Heparin (0,4 mg/ml) versetzt, zentrifugiert (5 Min., 4500 × *g*) und die Erythrozyten nach Abtrennung des Plasmas und Absaugen der Leukozyten- und Thrombozyten-haltigen Schicht zweimal im dreifachen Volumen einer Phosphat-LOCKE-Lösung[5] gewaschen.
Dann wurden die gewaschenen Erythrozyten im eigenen Plasma entsprechend einem Hämatokrit-Wert von 40% unter Zusatz von Glucose (10—20 µMol/ml Ansatz) resuspendiert. Das Plasma war jeweils mit empirisch ermittelten Mengen von 1N HCl soweit angesäuert, daß sich nach Zugabe der Erythrozyten ein pH-Wert von 7,35 einstellte. Zur Inkubation in Glucose-freien Medien wurden die Erythrozyten zweimal im 10fachen und einmal im 3fachen Volumen einer Glucose-freien Phosphat-LOCKE-Lösung gewaschen und anschließend in der gleichen Lösung ebenfalls entsprechend einem Hämatokrit von 40% suspendiert. Unter diesen Bedingungen betrug die endgültige Glucose-Konzenzentration weniger als 0,05 µMol/ml Ansatz.
Die Erythrozyten-Suspensionen wurden zunächst für 15—30 Min. bei 37°C vorinkubiert und anschließend auf mehrere Inkubationsgefäße verteilt, welche die zu prüfenden Schwefel-Verbindungen (bzw. Natriumchlorid in den Kontrollen) in isotonischer Lösung (pH

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[6] G. SAUER u. D. SCHOLZ, Folia haematol. [Leipzig] 83, 377 [1965].

7,0–7,5) enthielten. Die angegebenen Konzentrationen beziehen sich jeweils auf die Volumeneinheit Gesamtansatz (Erythrozyten + Medium).

Die Inkubation der Versuchsansätze erfolgte in geschlossenen Polyäthylenröhrchen (50 m*l* Fassungsvermögen, Luftatmosphäre) unter gleichmäßigem Schütteln (40/Min.) im Wasserbad bei 37°C. Während der Inkubationszeit (bis zu 8 Std.) kam es zu keiner nennenswerten Hämolyse. Der pH-Wert der Ansätze wurde während der gesamten Versuchsdauer mittels einer Einstab-Meßkette kontrolliert und durch Zugabe von 0,3N NaOH im Bereich von pH 7,27–7,37 konstant gehalten. Zu Beginn und Ende des Versuchs wurde der Hämatokrit-Wert mit einer CLAY-ADAMS-Autokrit-Zentrifuge (10 Min. bei 15000 × *g*) bestimmt.

Versuche an Hämolysaten

Frisch entnommene und zweimal im 3fachen Volumen Glucose-freier Phosphat-LOCKE-Lösung gewaschene Erythrozyten wurden in ein doppeltes Volumen eiskalten dest. Wassers eingegeben, 15 Min. bei 4°C gerührt und 20 Min. bei 20000 × *g* und 4°C zentrifugiert. Nach nochmaligem Zentrifugieren des Überstandes wurden die weitgehend von Erythrozyten-Stromata befreiten Hämolysate bei 37°C unter Einhaltung eines konstanten pH-Wertes von 7,2 bis zu 24 Std. mit und ohne Zusatz der schwefelhaltigen Verbindungen inkubiert.

Analysen

Nach verschiedenen Inkubationszeiten wurde jeweils ein Aliquot der Ansätze mit dem doppelten Volumen eiskalter 1,2N (Erythrozyten) bzw. 0,6N (Hämolysate) $HClO_4$ direkt enteiweißt und der Überstand nach Zentrifugieren durch Zugabe von 5N K_2CO_3 bzw. 6N KOH in der Kälte unter ständigem Rühren auf einen pH-Wert von 6,0 ± 0,5, für Pyruvat-Bestimmungen auf pH 3,5 gebracht. Die pH-Kontrolle erfolgte potentiometrisch mit einer Einstab-Meßkette. Nach 20 Min. Stehenlassen im Eisbad wurde der Kaliumperchlorat-Niederschlag abzentrifugiert und sofort mit der Aufarbeitung des Überstandes begonnen. Bei Experimenten mit intakten Erythrozyten wurde das Inkubationsmedium nach Abtrennung der Erythrozyten mit 10proz. Trichloressigsäure im Verhältnis 1:1 enteiweißt.

Orthophosphat, ATP und 2.3-Diphospho-glycerat wurden aus den Extrakten mittels zweidimensionaler Papierchromatographie isoliert[7] und ihre Konzentrationen nach feuchter Veraschung der Papierareale durch Phosphoranalysen quantitativ bestimmt[8].

Glucose, Pyruvat, ADP, AMP, 2- und 3-Phosphoglycerat, Phosphoenolpyruvat, Fructose-1.6-diphosphat, Dihydroxyacetonphosphat und Glycerinaldehyd-3-phosphat wurden enzymatisch unter Verwendung von Enzymen, Coenzymen und Substraten der Fa. Boehringer Mannheim GmbH nach Standard-Methoden analysiert[9]. Die Lactat-Bestimmung erfolgte mit geringen Modifikationen nach der Methode von GERCKEN[10]. Die chromatographischen und enzymatischen Bestimmungen wurden durch die Anwesenheit der Schwefel-Verbindungen in den Extrakten nicht gestört.

Die intrazellulären Konzentrationen von Phosphatverbindungen wurden jeweils unter Zugrundelegung des zu Inkubationsbeginn gemessenen Hämatokrit-Wertes berechnet und in µMol/m*l* Erythrozyten angegeben. Die intrazelluläre Orthophosphat-Konzentration wurde aus der Differenz zwischen dem Orthophosphat-Gehalt im Gesamtansatz und im Medium ebenfalls unter Zugrundelegung des Hämatokrit-Wertes ermittelt.

Ergebnisse

Einfluß anorganischer Schwefel-Verbindungen auf das Konzentrationsverhalten von 2.3-Diphosphoglycerat in intakten Erythrozyten und Hämolysaten

Während mehrstündiger Inkubation menschlicher Erythrozyten im eigenen Plasma kommt es auch bei ausreichendem Glucose-Angebot zu einer ursächlich bisher ungeklärten kontinuierlichen Konzentrationsverminderung von 2.3-Diphospho-glycerat[5,11]. In unseren Versuchen betrug die Geschwindigkeit der spontanen Abnahme des 2.3-Diphosphoglycerat-Spiegels ca. 0,17 µMol/m*l* Erythrozyten je Std. (s. Abb. 1); das entspricht einer Verminderung von etwa 30% in 8 Std.

Dithionit ($S_2O_4^{2\ominus}$) und Sulfit ($SO_3^{2\ominus}$; jeweils $5 \cdot 10^{-3}$M) beschleunigen den 2.3-Diphospho-glycerat-Abfall etwa auf das doppelte, Disulfit ($S_2O_5^{2\ominus}$) in der gleichen Konzentration auf das vierfache. Entgegengesetzt wirken Sulfat ($SO_4^{2\ominus}$) und Tetrathionat ($S_4O_6^{2\ominus}$), die in einer Konzentration von 5 bzw. $1 \cdot 10^{-3}$M den Abfall von 0,17 auf 0,03 µMol/m*l* Erythrozyten je Std. reduzieren (s. Abb. 1). Dies entspricht einer 80proz. Hemmung. Bei Erhöhung der Tetrathionat-Konzentration auf $5 \cdot 10^{-3}$M bleibt der 2.3-Diphospho-glycerat-Spiegel während 8stdg. Inkubation konstant. Ebenso wie Sulfat verzögert

[7] E. GERLACH, A. FLECKENSTEIN u. E. GROSS, Pflügers Arch. ges. Physiol. Menschen Tiere 266, 52 [1958].

[8] E. GERLACH u. B. DEUTICKE, Biochem. Z. 337, 477 [1963].

[9] H. U. BERGMEYER, Method. d. enzymat. Analyse, Verlag Chemie, Weinheim/Bergstr. 1962.

[10] G. GERCKEN, diese Z. 320, 180 [1960].

[11] R. WHITTAM, J. Physiology 140, 479 [1958]; B. D. GOMPERTS, Biochem. J. 102, 782 [1967].

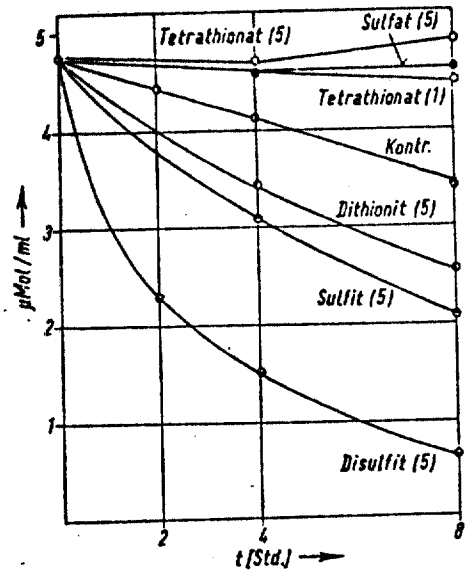

Abb. 1. Einfluß von anorganischen Schwefel-Verbindungen auf den spontanen Abfall der 2.3-Diphospho-glycerat-Konzentration in Menschen-Erythrozyten. Inkubation in Plasma (0,5% Glucose) bei pH 7,27 bis 7,37 und 37°C; Hämatokrit-Wert 40%; Mittelwerte aus jeweils 3—7 Versuchen. Die Konzentrationen der Zusätze sind in mMol/l an den Kurven angegeben. Ordinate: 2.3-Diphospho-glycerat-Konzentration [µMol/ml Erythrozyten].

auch Thiosulfat ($S_2O_3^{2\ominus}$) den 2.3-Diphospho-glycerat-Abfall.

In weiteren Studien wurde der Einfluß der verschiedenen Schwefel-Verbindungen auf den 2.3-Diphospho-glycerat-Abbau in Glucose-frei inkubierten Erythrozyten und Hämolysaten untersucht. Unter diesen Bedingungen hängt die Geschwindigkeit des 2.3-Diphospho-glycerat-Abfalls nur von der Aktivität der Diphosphoglycerat-Phosphatase ab, da eine 2.3-Diphospho-glycerat-Synthese nicht mehr erfolgt. Diese Versuche dienten einmal der Klärung der Frage, ob die Wirkung von Substanzen wie Pyrosulfit tatsächlich auf einer Aktivierung der Diphosphoglycerat-Phosphatase beruht[4,6]. Außerdem sollte geprüft werden, ob für die Verzögerung des 2.3-Diphospho-glycerat-Abfalls durch Tetrathionat und Sulfat eine Hemmung des Abbaus am Enzym, oder — wie im Falle von Dipyridamol[3,5] — eine erhöhte Synthese verantwortlich ist.

Die Resultate von Experimenten an Glucose-frei inkubierten Erythrozyten sind in Abb. 2 dargestellt.

Wie hieraus zunächst hervorgeht, ist die Geschwindigkeit des 2.3-Diphospho-glycerat-Abfalls in Abwesenheit von Glucose mit 0,58 µMol/ml Erythrozyten je Std. 3mal so hoch wie in Glucose-haltigen Medien. Ursache dieser Beschleunigung ist im wesentlichen die fehlende Neubildung von 2.3-Diphospho-glycerat[2]. Die Effekte der Schwefel-Verbindungen sind auch unter Substratmangel-Bedingungen nachweisbar, jedoch in geringerem Ausmaß als in glykolysierenden Erythrozyten. So wird beispielsweise der 2.3-Diphospho-glycerat-Abfall durch $5 \cdot 10^{-3}$M Sulfat bzw. $1 \cdot 10^{-3}$M Tetrathionat in Glucose-freien Erythrozyten nur zu ca. 25%, gegenüber 80% in Anwesenheit von Glucose, gehemmt.

Ähnlich liegen die Verhältnisse in Glucose-freien Hämolysaten (Abb. 3): Hier beträgt die Abbaurate unter Kontrollbedingungen 0,12 µMol 2.3-Diphosphoglycerat/ml Hämolysat je Std.; sie wird durch Disulfit etwa verdoppelt und durch Sulfat um 70%

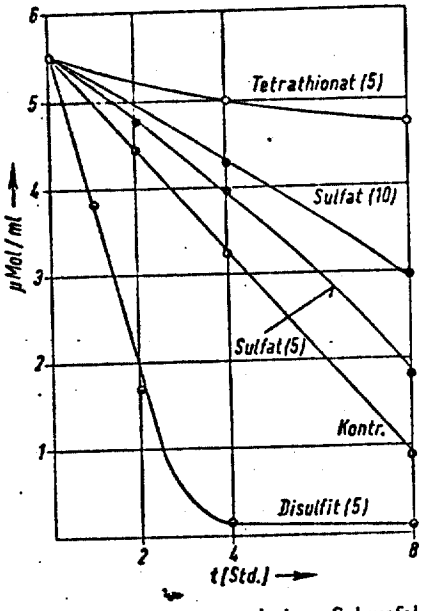

Abb. 2. Einfluß von anorganischen Schwefel-Verbindungen auf den spontanen Abfall der 2.3-Diphospho-glycerat-Konzentration in Menschen-Erythrozyten bei Inkubation ohne Glucose. Inkubation in Glucose-freier Phosphat-Lockt-Lösung bei pH 7,30—7,35 und 37°C; Hämatokrit-Wert 40%; Mittelwerte aus jeweils 3—5 Versuchen. Die Konzentrationen der Zusätze sind in mMol/l an den Kurven angegeben. Ordinate: 2.3-Diphospho-glycerat-Konzentration [µMol/ml Erythrozyten].

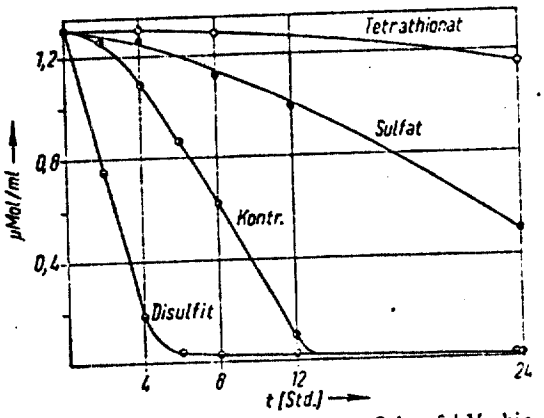

Abb. 3. Einfluß von anorganischen Schwefel-Verbindungen auf den Abbau von 2.3-Diphospho-glycerat in Glucose-freien Hämolysaten. Inkubation bei pH 7,2 und 37°C; Wirkstoff-Konzentrationen jeweils 5mM; Mittelwerte aus jeweils 3 Versuchen. Ordinate: 2.3-Diphospho-glycerat-Konzentration [µMol/ml Hämolysat].

vermindert. Tetrathionat ($5 \cdot 10^{-3}$M) verhindert sowohl in Glucose-freien intakten Erythrozyten als auch in Hämolysaten den Abfall nahezu vollständig. In speziellen Untersuchungen wurde die Wirkung von Disulfit auf den 2.3-Diphospho-glycerat-Abbau auch an einer Hämoglobin-freien Enzympräparation aus Menschen-Erythrozyten* geprüft. Hier beschleunigte Disulfit ($1 \cdot 10^{-2}$M) den Abbau um 45%.

Auf Grund all dieser Befunde läßt sich feststellen, daß Schwefel-Verbindungen den 2.3-Diphospho-glycerat-Abfall auch unter Bedingungen beeinflussen, unter denen keine 2.3-Diphospho-glycerat-Synthese stattfindet. Dies kann als Beweis dafür angesehen werden, daß die Verzögerung des 2.3-Diphospho-glycerat-Abfalls unter Tetrathionat auf einer Hemmung des Abbaus beruht. Dabei dürfte es sich um einen direkten Einfluß der Schwefel-Verbindung auf die Diphospho-glycerat-Phosphatase handeln. Die geringfügigen Veränderungen der 3-Phospho-glycerat-Konzentration (s. Tab. 2) reichen zur Erklärung des Hemmeffektes nicht aus[13].

Die Beschleunigung des Abfalls der 2.3-Diphospho-glycerat-Konzentration durch Disulfit, Sulfit und Dithionit ist dementsprechend auf einen gesteigerten Abbau zurückzuführen. Dieser Abbau erfolgt sicherlich auf dem Weg über 3-Phospho-glycerat. Als Abbauprodukte reichern sich allerdings — wie schon früher von MÁNIAY und VÁRADY[4] gezeigt wurde — nur Pyruvat und anorganisches Phosphat an (vgl. Tab. 1 und 2). Die Konzentrationen von 3- und 2-Phospho-glycerat sowie Phosphoenolpyruvat ändern sich nur geringfügig, da die Aktivitäten der Phosphoglyceromutase, Phosphopyruvat-Hydratase und Pyruvat-Kinase[14] im Erythrozyten sehr hoch sind.

Für eine Aktivierung des normalen Abbauweges von 2.3-Diphospho-glycerat durch die Schwefel-Verbindungen spricht außerdem, daß der durch Disulfit induzierte ebenso wie der normale Abbau durch Fluorid verhindert wird[15]. Fluorid hemmt die Phosphopyruvat-Hydratase und verursacht dadurch eine starke Konzentrationserhöhung von 3-Phospho-glycerat, das seinerseits die Diphospho-glycerat-Phosphatase hemmt[16].

In eigenen Versuchen erhöhte sich bei Zusatz von Fluorid (8mM) zu Disulfit-behandelten (10mM) Erythrozyten die 3-Phospho-glycerat-Konzentration von 0,1 auf 1,1 µMol/ml Erythrozyten, während die Geschwindigkeit des 2.3-Diphospho-glycerat-Abfalls von 1,76 auf 0,18 µMol/ml Erythrozyten je Std. abnahm. Diese Aufhebung des Disulfit-Effektes infolge Erhöhung der 3-Phospho-glycerat-Konzentration spricht dafür, daß die Disulfit-Wirkung auf einer direkten Aktivierung der Diphosphoglycerat-Phosphatase beruht.

Einfluß anorganischer Schwefel-Verbindungen auf die Glykolyse des Menschen-Erythrozyten

a) Einfluß auf Glucose-Verbrauch und Lactat-Bildung

Früheren Befunden zufolge werden Glucose-Verbrauch und Lactat-Bildung des Menschen-Erythro-

* Fällung des Hämoglobins mit Äthanol/Chloroform nach osmotischer Hämolyse[12]; Entfernung von Äthanol und Chloroform sowie Einengung auf das Ausgangsvolumen der Erythrozyten durch Evaporation bei 30°C; Zusatz von 50mM Triäthanolamin, 1mM Monojodessigsäure, 1mM 2.3-Diphospho-glycerat, 3,5mM ADP, Pyruvat-Kinase, Phosphopyruvat-Hydratase und Phosphoglyceromutase je 100 mg/ml; Inkubation bei pH 7,2 und 37°C.

[12] T. HASHIMOTO u. H. YOSHIKAWA, J. Biochemistry [Tokyo] 56, 279 [1964].

[13] C.-H. DE VERDIER, Folia haematol. [Leipzig] 90, 215 [1968].

[14] G. W. LÖHR u. H. D. WALLER, Klin. Wschr. 37, 833 [1959]; F. GRIGNANI u. G. W. LÖHR, Klin. Wschr. 38, 796 [1960].

[15] G. GÁRDOS, Acta biochim. biophys. Acad. Sci. hung. 1, 139 [1966].

[16] S. RAPOPORT u. J. LUEBERING, J. biol. Chemistry 183, 507 [1950]; 189, 683 [1951]; C.-H. DE VERDIER, Biochem. J. 92, 38P [1964]; Z. B. ROSE, J. biol. Chemistry 243, 4810 [1968].

Tab. 1. Veränderungen der Glykolyse-Rate und der Geschwindigkeit des 2.3-Diphospho-glycerat-Abfalls in Menschen-Erythrozyten unter dem Einfluß verschiedener Schwefel-Verbindungen ($5 \cdot 10^{-3}$M). (Werte in µMol/ml Erythrozyten je Std. ± Standardabweichung).

	Zeit [Std.]	Glucose-Verbrauch	Lactat-Bildung	Konzentrationsänderung von Pyruvat	Konzentrationsänderung von 2.3-Diphospho-glycerat
Kontrolle (n=6)	0—4	1,48 ± 0,18	2,76 ± 0,35	+0,05 ± 0,03	−0,14 ± 0,04
	4—8	1,40 ± 0,21	2,63 ± 0,20	+0,08 ± 0,02	−0,14 ± 0,04
Tetrathionat (n=4)	0—4	3,01 ± 1,06	3,58 ± 1,14	+0,02 ± 0,02	−0,05 ± 0,04
	4—8	1,93 ± 0,48	3,52 ± 0,85	+0,01 ± 0,02	+0,07 ± 0,03
Thiosulfat (n=3)	0—4	3,07 ± 0,75	4,58 ± 0,92	−0,02 ± 0,06	−0,01 ± 0,01
	4—8	2,48 ± 0,36	4,24 ± 0,63	−0,01 ± 0,02	0,00 ± 0,01
Sulfat (n=4)	0—4	2,84 ± 0,49	4,27 ± 0,47	−0,01 ± 0,02	−0,03 ± 0,05
	4—8	2,02 ± 0,11	4,10 ± 0,49	−0,01 ± 0,02	+0,07 ± 0,05
Disulfit (n=4)	0—4	3,27 ± 0,64	5,31 ± 1,17	+0,78 ± 0,12	−0,71 ± 0,07
	4—8	3,13 ± 0,38	5,53 ± 0,44	+0,31 ± 0,20	−0,29 ± 0,01
Sulfit (n=3)	0—4	2,44 ± 0,47	4,53 ± 1,01	+0,49 ± 0,03	−0,39 ± 0,04
	4—8	2,52 ± 0,40	4,37 ± 0,64	+0,10 ± 0,26	−0,27 ± 0,10
Dithionit (n=3)	0—4	2,94 ± 0,46	4,86 ± 0,80	+0,42 ± 0,04	−0,34 ± 0,03
	4—8	2,73 ± 0,40	5,05 ± 0,75	−0,02 ± 0,23	−0,27 ± 0,03

zyten durch Sulfit[4,17] und Sulfat[18] gesteigert. Um zu klären, ob auch die anderen von uns untersuchten Schwefel-Verbindungen eine derartige Wirkung haben, wurde ihr Einfluß auf Glykolyse-Rate und Metabolit-Konzentrationen im Erythrozyten analysiert.

Wie aus Tab. 1 hervorgeht, erhöhen alle Inhibitoren und Aktivatoren des 2.3-Diphospho-glycerat-Abbaus Glucose-Verbrauch und Lactat-Bildung der Erythrozyten um etwa 50—100%. In Gegenwart von Disulfit, Dithionit und Sulfit kommt es außerdem zu einer beträchtlichen Anhäufung von Pyruvat. Die Pyruvat-Bildung entspricht dabei weitgehend der Abbaurate von 2.3-Diphospho-glycerat.

Bei Vergleich der Meßwerte für Glucose-Verbrauch und Lactat-Bildung zeigt sich, daß in Gegenwart aller Schwefel-Verbindungen die Lactat-Bildung geringer ist, als der Glucose-Verbrauch erwarten läßt. Ursache dieses Lactat-Defizits ist eine Anhäufung Phosphat-haltiger Intermediärprodukte der Glykolyse, und zwar im wesentlichen von Triosephosphaten (Dihydroxyacetonphosphat, Glycerinaldehyd-3-phosphat) und Fructose-1.6-diphosphat (vgl. Tab. 2). Tetrathionat und Disulfit verursachen daneben auch eine geringe Erhöhung des 3-Phosphoglycerat- und Phosphoenolpyruvat-Spiegels. Die Konzentrationsveränderungen der Triosephosphate und von Fructose-1.6-diphosphat bleiben nicht konstant. Nach 8 Std. beträgt in Gegenwart von Sulfat und Tetrathionat die Summe dieser Verbindungen nur noch etwa die Hälfte des Wertes nach 4stdg. Inkubation. Der Quotient Dihydroxyacetonphosphat + Glycerinaldehyd-3-phosphat/Fructose-1.6-diphosphat steigt während dieser Zeit an. Als Folge der jeweiligen Veränderung der Pyruvat-Bildung nimmt der Lactat/Pyruvat-Quotient unter Tetrathionat und Sulfat zu, in Gegenwart von Disulfit dagegenerheblich ab (s. Tab. 1 u. 2).

b) *Einfluß auf denATP-Spiegel*

Wie Tab. 3 zeigt, vermindert sich unter Tetrathionat- und Sulfat-Einwirkung der ATP-Spiegel um 45 bzw. 15%, ohne daß sich der ATP/ADP-Quotient wesentlich ändert. Die Summe der Adenin-Nucleotide nimmt dagegen erheblich ab. Eine nennens-

[17] J. C. Parker, J. clin. Invest. 48, 117 [1969].

[18] S. C. Rizzo u. R. E. Eckel, Amer. J. Physiol. 211, 429 [1966].

Tab. 2. Einfluß von Tetrathionat, Sulfat und Disulfit ($5 \cdot 10^{-3}$M) auf die Konzentrationen verschiedener Intermediärprodukte der Glykolyse in Menschen-Erythrozyten. (Werte in µMol/ml Erythrozyten ± Standardabweichung).

	Zeit [Std.]	Fru-1.6-P_2	Dihydroxy-acetonphosphat + Glycerinaldehyd-3-phosphat	3-*P*-Glycerat	2-*P*-Glycerat	*P*-Enolpyruvat	Lactat / Pyruvat	P_i intrazell.	P_i extrazell.
Kontrolle (n=6)	0	0,003 ± 0,002	0,016 ± 0,006	0,063 ± 0,013	0,005 ± 0,002	0,031 ± 0,011	36,3 ± 13,3	0,76 ± 0,10	0,99 ± 0,22
	4	0,004 ± 0,002	0,021 ± 0,004	0,070 ± 0,015	0,007 ± 0,003	0,033 ± 0,009	39,6 ± 6,6	0,93 ± 0,18	1,52 ± 0,30
	8	0,003 ± 0,001	0,029 ± 0,010	0,064 ± 0,020	0,005 ± 0,002	0,032 ± 0,011	35,9 ± 5,3	1,30 ± 0,35	2,01 ± 0,43
Tetrathionat (n=3)	4	0,388 ± 0,196	0,80 ± 0,18	0,122 ± 0,049	0,009 ± 0,006	0,070 ± 0,006	60,3 ± 5,0	0,48 ± 0,32	1,14 ± 0,29
	8	0,150 ± 0,049	0,50 ± 0,09	0,084 ± 0,015	0,007 ± 0,002	0,069 ± 0,019	106,5 ± 25,0	0,94 ± 0,09	1,34 ± 0,14
Sulfat (n=4)	4	1,12 ± 0,55	1,36 ± 0,44	0,045 ± 0,014	0,005 ± 0,001	0,033 ± 0,006	76,8 ± 10,2	0,44 ± 0,14	0,40 ± 0,13
	8	0,24 ± 0,11	0,75 ± 0,16	0,073 ± 0,010	0,007 ± 0,002	0,051 ± 0,014	107,8 ± 26,2	0,75 ± 0,48	1,17 ± 0,61
Disulfit (n=4)	4	0,118 ± 0,084	0,92 ± 0,24	0,091 ± 0,009	0,008 ± 0,002	0,052 ± 0,011	7,6 ± 1,5	2,09 ± 0,22	3,16 ± 0,27
	8	0,078 ± 0,033	0,73 ± 0,15	0,094 ± 0,018	0,004 ± 0,002	0,053 ± 0,002	11,5 ± 1,8	2,46 ± 0,65	4,15 ± 0,50

werte IMP-Bildung wurde dabei nicht beobachtet. Demnach kommt es in Gegenwart von 5 mM Tetrathionat oder Sulfat zu einem Abbau der Adenin-Nucleotide über die Monophosphat-Stufe hinaus. Ursache des ATP-Schwundes dürfte ein temporärer Orthophosphat-Mangel sein, der infolge des stark erhöhten Einbaus von anorganischem Phosphat in organische Bindung zu Beginn der Inkubation mit Tetrathionat oder Sulfat auftritt. Für die Richtigkeit dieser Annahme spricht sowohl die Beobachtung, daß sich der Sulfat-bedingte ATP-Abbau durch Zusatz von Orthophosphat verhindern läßt[18], als auch der Befund, daß Disulfit, welches den zellulären Orthophosphat-Spiegel auf Kosten von 2.3-Diphospho-glycerat stark erhöht, die ATP-Konzentration unbeeinflußt läßt, obwohl es die Glykolyse ebenso stimuliert wie Sulfat und Tetrathionat.

Diskussion

Der spontane Konzentrationsabfall von 2.3-Diphospho-glycerat in glykolysierenden und Glucose-freie inkubierten Erythrozyten sowie in Hämolysaten wird durch Dithionit, Sulfit und Disulfit erheblich beschleunigt. Verantwortlich für diesen Effekt ist — wie von MÁNIAY und VÁRADY postuliert wurde[1] — eine direkte Aktivierung der Diphosphoglycerat-Phosphatase. Diese Auffassung wird durch die vorliegenden Resultate gestützt: Einerseits erwiesen sich die genannten Substanzen auch nach Unterbrechung der 2.3-Diphospho-glycerat-Synthese als wirksam, andererseits nahm in ihrer Gegenwart die Konzentration von 3-Phospho-glycerat, einem Inhibitor der Diphosphoglycerat-Phosphatase[16], nicht ab (vgl. Tab. 2). Die Hemmbarkeit dieses Enzyms durch 3-Phospho-glycerat bleibt in Gegenwart von Disulfit erhalten.

Disulfit wirkt scheinbar doppelt so stark wie die beiden anderen Aktivatoren. Dies beruht jedoch nur darauf, daß sich Disulfit in verdünnten wäßrigen Lösungen fast quantitativ entsprechend den Reaktionen $S_2O_5^{2\ominus} + H_2O \rightarrow 2\ HSO_3^{2\ominus}$; und $HSO_3^{2\ominus} \rightleftarrows SO_3^{2\ominus} + H^{\oplus}$ ($K' = 1 \cdot 10^{-7}$) umwandelt[19].

Unsere Befunde stehen im Widerspruch zu Angaben von SAUER und SCHOLZ[6], die an Hämolysaten und an einem gereinigten Diphosphoglycerat-Phosphatase-Präparat aus Kaninchen-Erythrozyten

[19] GMELINS Handb. d. anorgan. Chemie, 8. Aufl. Bd. 9, B 2, S. 456ff., Verlag Chemie, Weinheim/Bergstr. 1960.

Tab. 3. Einfluß von Tetrathionat, Sulfat und Disulfit ($5 \cdot 10^{-3}$M) auf die Konzentrationen von Adenin-Nucleotiden in Menschen-Erythrozyten. (Werte in µMol/ml Erythrozyten ± Standardabweichung).

	Zeit [Std.]	ATP	ADP	AMP	$\sum$ Adenin-Nucleotide	ATP/ADP
	0	1,26 ± 0,07	0,21 ± 0,02	0,07 ± 0,01	1,55 ± 0,08	6,1 ± 0,7
Kontrolle (n=5)	4	1,29 ± 0,07	0,18 ± 0,02	0,07 ± 0,01	1,53 ± 0,10	7,5 ± 0,7
	8	1,28 ± 0,04	0,17 ± 0,03	0,07 ± 0,01	1,52 ± 0,06	7,9 ± 1,2
Tetrathionat (n=3)	4	0,73 ± 0,07	0,07 ± 0,01	0,03 ± 0,02	0,81 ± 0,06	10,3 ± 1,2
	8	0,65 ± 0,11	0,08 ± 0,02	0,03 ± 0,02	0,79 ± 0,11	8,5 ± 3,2
Sulfat (n=3)	4	0,98 ± 0,24	0,18 ± 0,05	0,09 ± 0,01	1,31 ± 0,21	6,1 ± 3,1
	8	0,98 ± 0,22	0,14 ± 0,02	0,07 ± 0,02	1,24 ± 0,24	7,6 ± 2,4
Disulfit (n=3)	4	1,31 ± 0,06	0,11 ± 0,04	0,06 ± 0,01	1,48 ± 0,09	12,6 ± 4,2
	8	1,30 ± 0,01	0,17 ± 0,05	0,10 ± 0,03	1,57 ± 0,02	7,9 ± 2,0

keine Aktivierung durch Sulfit nachweisen konnten. Sie finden jedoch eine Bestätigung durch Resultate von HARKNESS und ROTH, die in neuester Zeit zeigten, daß ein aus Menschen-Erythrozyten gereinigtes Enzympräparat durch Disulfit und Dithionit aktiviert wird[20].

Ebenfalls auf einer direkten Beeinflussung der Diphosphoglycerat-Phosphatase beruht die Hemmung des 2.3-Diphospho-glycerat-Abbaus durch Tetrathionat, Thiosulfat und Sulfat. Auch diese Verbindungen sind in Hämolysaten wirksam, ohne den 3-Phospho-glycerat-Spiegel wesentlich zu beeinflussen.

Die Hemmung des Enzyms durch Sulfat ist auch unter pathophysiologischen Aspekten von Interesse: Bei Urämien und chronischen Nephritiden wurden von verschiedenen Autoren erhöhte 2.3-Diphospho-glycerat-Konzentrationen im Erythrozyten beobachtet[21], für die es bisher keine Erklärung gab. Da bei urämischen Zuständen der Sulfat-Spiegel im Plasma, der normalerweise ca. 0,5mM beträgt, bis auf 8mM ansteigen kann[22], liegt die Vermutung nahe, daß die Konzentrationserhöhung von 2.3-Diphospho-glycerat unter derartigen Verhältnissen durch eine Sulfat-bedingte Hemmung des Abbaus bei unveränderter Synthese verursacht wird.

Die Glykolyse des Menschen-Erythrozyten wird durch alle von uns untersuchten Schwefel-Verbindungen gleichartig und annähernd gleich stark beeinflußt: Es kommt zu einer deutlichen Steigerung des Glucose-Verbrauchs, begleitet von einer quantitativ nicht entsprechenden Zunahme der Lactat-Bildung und einer charakteristischen Anhäufung verschiedener Metabolite, insbesondere von Fructose-1.6-diphosphat, Dihydroxyacetonphosphat und Glycerinaldehyd-3-phosphat. Unsere Befunde stimmen hinsichtlich des Einflusses von Sulfat und Disulfit mit Resultaten von ECKEL und RIZZO sowie von PARKER weitgehend überein[17, 18].

Die Metabolit-Veränderungen in Gegenwart der Schwefel-Verbindungen entsprechen im wesentlichen denjenigen, die bei Stimulation der Glykolyse durch Phosphat-Zusatz oder pH-Erhöhung gefunden werden. In diesen Fällen ist eine Aktivierung der Phosphofructokinase Ursache der Veränderungen[18, 23]. Es liegt daher nahe, die Glykolysesteigerung in Gegenwart von Tetrathionat, Thiosulfat, Dithionit und Sulfit ebenfalls auf eine Aktivierung dieses Enzyms zurückzuführen. Eine direkte Beeinflussung der Phosphofructokinase durch Sulfat konnte von ECKEL und RIZZO bereits früher nachgewiesen werden[18].

Ein spezielles Problem ergibt sich aus dem Befund,

[20] D. R. HARKNESS u. S. ROTH, Biochem. biophys. Res. Commun. 34, 849 [1969].

[21] G. M. GUEST u. S. RAPOPORT, Physiol. Rev. 21, 410 [1941]; H. JAHRMÄRKER, Klin. Wschr. 42, 123 [1964]; G. A. HURT u. A. CHANUTIN, J. Lab. clin. Med. 64, 675 [1964].

[22] A. STURM u. A. POTHMANN, Z. klin. Med. 137, 467 [1940]; E. MILLER, C. J. HEAD, M. S. STANLEY LEVINE, J. H. HOLMES u. H. ELRICK, J. Lab. clin. Med. 58, 656 (1961); S. HÄNZE, Klin. Wschr. 44, 1247 [1966].

[23] S. MINAKAMI, T. SAITO, C. SUZUKI u. H. YOSHIKAWA, Biochem. biophysic. Res. Commun. 17, 748 [1964]; K. K. TSUBOI u. K. FUKUNAGA, J. biol. Chemistry 240, 2806 [1965]; I. A. ROSE u. J. V. B. WARMS, J. biol. Chemistry 241, 4848 [1966].

daß Tetrathionat die Glykolyse des Menschen-Erythrozyten aktiviert. Tetrathionat ist nämlich ein sehr wirksamer Hemmstoff der Glycerinaldehyd-3-phosphat-Dehydrogenase[24]. Wenn die Glykolyse trotzdem nicht gehemmt wird, so dürfte dies entweder darauf zurückzuführen sein, daß das Enzym im intakten Erythrozyten vor der Hemmwirkung von Tetrathionat geschützt ist, oder aber darauf beruhen, daß Tetrathionat in den Erythrozyten ebensowenig eindringt wie in andere Zellen[25]. Im letzteren Falle wäre dann allerdings die Hemmung der Diphosphoglycerat-Phosphatase und die Aktivierung der Phosphofructokinase in intakten Erythrozyten nur mit einem transmembranen Effekt von Tetrathionat oder einer Lokalisation beider Enzyme in der Membran zu erklären. Nicht auszuschließen ist allerdings die Möglichkeit, daß Tetrathionat während der Inkubationszeit in eine Verbindung umgewandelt wird, die zwar die Diphosphoglycerat-Phosphatase und Phosphofructokinase, nicht aber die Glycerinaldehyd-3-phosphat-Dehydrogenase beeinflußt. Über chemische Umsetzungen der von uns geprüften Schwefel-Verbindungen in Erythrozyten bzw. Plasma ist unseres Wissens bisher nichts bekannt.

Über die Mechanismen, die den verschiedenen Wirkungen der Schwefel-Verbindungen zu Grunde liegen, sind noch keine eindeutigen Aussagen möglich. Es erscheint jedoch denkbar, daß die Aktivierung der Phosphofructokinase — ebenso wie im Falle von Phosphat — auf einer Kompetition der aktivierenden Anionen mit ATP und einer hierdurch verursachten Aufhebung der ATP-bedingten Hemmung des Enzyms beruht[26]. Die reduzierende Wirkung einiger Aktivatoren ($SO_3^{2\ominus}$, $S_2O_3^{2\ominus}$ und $S_2O_4^{2\ominus}$) dürfte nichts mit der Stimulation zu tun haben, denn andere Aktivatoren ($PO_4^{3\ominus}$, $SO_4^{2\ominus}$ und $S_4O_6^{2\ominus}$) haben diese Eigenschaften nicht.

Vorläufig nicht zu beantworten ist die Frage, warum die Diphosphoglycerat-Phosphatase durch Sulfit und Dithionit aktiviert, durch Sulfat, Tetrathionat und Thiosulfat gehemmt wird. Sucht man nach gemeinsamen Eigenschaften der Aktivatoren, so zeigt sich, daß sie alle starke Reduktionsmittel sind, die beispielsweise Disulfidbrücken unter Bildung von SH-Gruppen und gemischten Disulfiden reduzieren. Die Beteiligung eines derartigen Effektes an der Aktivierung dieses Enzyms erscheint jedoch wenig wahrscheinlich, da die Spaltung von Disulfidbrükken im allgemeinen mit einer Inaktivierung von Enzymen einhergeht[27]. Außerdem haben neuere Untersuchungen von Rose ergeben, daß auch Phosphat die Diphosphoglycerat-Phosphatase aktiviert[28]. Die Hemmstoffe des Enzyms weisen keine gemeinsamen chemischen Eigenschaften auf, wenn man davon absieht, daß sie im Gegensatz zu den Aktivatoren +6-wertige Schwefel-Atome enthalten.

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