Phosphates A Monograph 1/26/73

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PHOSPHATES

A Monograph

Prepared for

Department of Health, Education and Welfare

Public Health Service

Food and Drug Administration

Rockville, Maryland

by

Dr. Paul C. Walter

Dr. Jon E. Villaume and

Dr. Thomas J. Taylor

FIRL Project No. 80G-C3378-01

January 26, 1973

THE FRANKLIN INSTITUTE RESEARCH LABORATORIES

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SUMMARY

The origin of mammalian host phosphorus pools is mainly exogenous. Accordingly, portal of entry differences are decisive for hydrolytic outcomes. Metabolic acidosis and hypocalcemia are the mutually exhaustive, but not mutually exclusive, biochemical origins of the lethal or sub-lethal effects reported for phosphoric acid and its salts. The occurrence of each is uniquely related to the rate of condensed phosphate hydrolysis.

Relevant abbreviations are as follows: orthophosphate - oP, pyrophosphate - PP, tripolyphosphate - TPP, hexametaphosphate - HMP, trimetaphosphate - c-triMP and tetrametaphosphate - c-tetMP. Unless otherwise indicated, oP is construed as a mixture of NaH_2PO_4 and Na_2HPO_4.

Toxicity and Hydrolysis

Orthosphosphate and adenosine monophosphate, only, were recovered in rat plasma after sodium PP administration by gavage, suggesting that a p.o. polyphosphate effect was equivalent to that of orthophosphate alone (270). The i.p. LD_{50} in decreasing order was PP, TPP, sodium HMP, oP, calcium HMP, c-tetMP and c-triMP for the rat (323,324). The descending order (TPP>HMP>c-triMP) was confirmed by long-term dietary supplementation in rats and dogs (33) and in p.o. and s.c. studies in the mouse (1012). In the latter study, the i.v. LD_{50} was 5-25x lower than that of the s.c., while the oral LD_{50} 4-7x and 40-100x higher, resp., than that for s.c. and i.v. This order of magnitude difference between the oral and parenteral result was attributed either to negligible gut resorption and concomitant nonspecific osmotic salt effects or else complete gastrointestinal hydrolysis with orthophosphate resorption only. Given that membrane transit of partially hydrolyzed linear polyphosphates or cyclic oligomers is at a competitive disadvantage (445), oral toxicity of phosphates in general would then be a function of orthophosphate absorption, distribution and accumulation uniquely. Even PP p.o. was never identified in urine or feces (218), and there was evidence that oP and PP compete for the same enzyme site (652).

Tracer Distribution

There was little difference between labeled oP and PP intraluminal fates except in rate of enteral passage. Label harvest from colon contents of each agent was equivalent by 2 h post-gavage, but gastric evacuation of oP was much slower - 3x more PP passing the pylorus between 30 and 60 min post-treatment. This rate was confirmed by label recovery from gastrointestinal mucosa. Colon lumen accumulation of HMP was a function of time, perhaps suggesting some nonspecific binding between gut mucosa and polyphosphate chain. The decrements in gastrointestinal mucosal label for HMP, especially after 1 hour, would also suggest excretion into the lumen after absorption (74). Phosphorus partition studies of gut lumen contents found increasingly larger amounts of calcium and phosphorus during intestinal descent. Gastric emptying time was unaffected by Na_2HPO_4 (873). Na_3PO_4 was relatively ineffective as a gastric secretagogue, although increasing dietary phosphate concentrations eliciting graded secretory rate responses with plateau formation would suggest a gradient between gastric lumen content and gastric mucosa (662). Liver, serum and kidney label recovery data also varied insignificantly between oP and PP, with only trace evidence of HMP label. Maxima were reached at 2 h post-treatment and subsided very slowly. Tibial label increased exponentially up to 6 h after both oP and PP (74). This contrasts with p.o. data on H_3PO_4, $CaHPO_4$ and $Ca_3(PO_4)_2$ caused deposition in liver, kidney, muscle and femur, where H_3PO_4 label accumulation was significantly greater than the salts in rats at 2 h maxima, with subsequent proportional decreases (43). Infusion i.v. of $H_3{}^{32}PO_4$ in rabbits elicited a ductus choledochus label maximum 10-15 min post-treatment, with exponential decline (626).

Renal and Intestinal Clearance

^{32}P recovery in urine after gavage delivery of H_3PO_4 in rats found 8% of the dose recovered by 72h, with twice that value for $CaHPO_4$, $Ca_3(PO_4)_2$ and $Ca(H_2PO_4)_2$. Although there was greater tissue absorption and retention of acid label, most of the label eliminated from each agent by 72h was already excreted by 48h (43). There was

negligible increase in serum oP, with exponential removal from blood, prompt accumulation of unhydrolyzed fraction in urine and complete renal elimination by 4 hours after i.v. administration of sodium c-triMP and c-tetMP (25mgP/kg) in rabbits (324). Urinary oP was less than 2% of unhydrolyzed phosphates. At least twice the i.p. load in rats elicited 90% unhydrolyzed cyclic metaphosphate in urine after 24 hr with no increase in oP. When the calcium homologs were used, the unhydrolyzed fraction in urine was 50-75%, with some increase in urinary oP. HMP and TPP in rat and rabbit were significantly hydrolyzed, with 2x as much oP in urine as unhydrolyzed phosphorus. High amounts of oP, with no polymeric phosphate pool, were recovered in rat and rabbit urine after p.o. delivery of cyclic or linear condensed phosphates, indicating at least the presence of trimetaphosphatase activity and gastrointestinal hydrolysis of cyclic oligmers (324). HMP conversion to oP without PP formation had been proposed long ago (479).

Hypocalcuric states were reported after p.o. administration of orthophosphate and orthophosphoric acid (696) without change in calcium balance, potassium or nitrogen turnover or fixed bases but with lower urinary pH and increased NH_3 excretion (578). Renal excretion of sodium and potassium rose 100 and 150%, resp., while chloride elimination fell in humans receiving 1% orthophosphate(317). Acid excretion was nearly 50% higher than controls within the first day following 29 mg P/kg i.p. sodium HMP in rats, a dose 163 mg P/kg below the LD_{50}. The rate of acid formation was still sufficiently high to elicit a moderate systemic acidosis, as measured by the rapid 35-40% fall in arterial CO_2(323). The extremely slow normalization of plasma CO_2 levels would also suggest renal elimination of fixed base.

The relative amounts of ^{32}P recovered in rat feces, as percent of dose, after p.o. (gavage) was 12, 31, 50 and 43% at 72 h, resp., for H_3PO_4, $CaHPO_4$, $Ca_3(PO_4)_2$ and $Ca(H_2PO_4)_2$. As with urinary outcomes, there was significantly higher absorption and retention of label from the acid than the salts and 90-95% of total label eliminated had been excreted by 48 hours (43). $(NH_4)_2HPO_4$ supplementation in high starch diets to rats with negative calcium balance showed no

relation between oral intake and colon clearance of phosphorus up to a threshold of ca. 20mg. A positive covariation might obtain at higher inputs (above 20mg), which would suggest absorption of a constant amount of phosphorus independently of amount ingested. Evidence that fecal phosphorus elimination can exceed simultaneous phosphorus intake, if the intake is sufficiently low, is supportive of a resorption limit. In contrast, there was a discriminable tendency for urinary phosphorus to vary with intake and with no other factor. Above some extrapolated value of ca. 6 mg urinary phosphorus (corresponding to the intake of phosphorus-free rations), the amount of phosphorus ingested was urinated practically quantitatively (975). Label studies of dietary PP have identified an 80 and 20% recovery, resp., in urine and feces as oP (218).

Growth Response and Reproductive Effects

Na_2HPO_4 supplementation (6.3 g P/kg feed) in cattle for 6-7 months was growth optimal (943). Body weight loss in rats after 3-5% diet supplementation by oP, PP, TPP and HMP varied directly with percent in the ration above a certain non-responsive threshold (ca. 1.8% for all salts) (355). Growth retardation occurred in rats on 10% HMP, TPP c-triMP or c-tetMP supplements in long-term studies (33).

Mean liver, kidney and spleen weights were increased after 3-5% oP, TPP, PP or HMP additions to rat diets (355). Sodium phosphate i.p. in rabbits and i.v. in dogs for 3-8 weeks led to parathyroid hypertrophy (720). Parathyroid increases of 295 and 235% followed p.o. ingestion of 300-700 mg/kg NaH_2PO_4 and $(NH_4)_2HPO_4$, resp., by guinea pigs for 5-16 months, a conceivable outcome from acidosis (S-2). Thymus mitotic rate increased in irradiated and unirradiated rats after oP treatment (707). Adrenal weight decreased after NaH_2PO_4 treatment by gavage 2x daily for 12 days in rats and adrenal noradrenalin secretion was increased (322). Sodium phosphate with hydrocortisone increased, while desoxycortisone supplementation of the combination decreased, adrenal weight. Relative splenomegaly was reported for 5% dietary TPP, HMP and c-tetMP, in descending order, after a 2-year feeding in rats (33). Liver, testes, brain, stomach and heart were also enlarged after TPP, but in males only. Femur length was lower for both sexes,

with evidence of hydropexis and less organic material in long bone. A 30 mg P/kg dose of NaH_2PO_4 i.p. elicited increases of 58% in muscle phosphocreatine, 18% in muscle lactic acid, with normalization by 24h (591). This would imply a direct phosphate effect on muscle creatine-phosphocreatine balance with subsequent phosphocreatine accumulation. K_2HPO_4 in necrogenic rat rations significantly increased mean survival time and was prophylactic of diet-associated deficits in oxidative phosphorylation (437). Chromatid breaks under anaerobic conditions with 0.002M PP were attributed to inhibition of RNA and DNA synthesis (644). Sperm cell motility was inhibited by incubation with 100 ppm sodium metaphosphate, which effect was irreversible after a plateau was reached at 45 min (44). Total ^{32}P label in ewe milk fed ammonium phosphate diets and receiving ^{32}P intraruminally peaked at 21 hr, concurrent with serum label. A similar milk label was found after $CaHPO_4$ administration (723). No effect on fertility, litter size, growth survival, organ weight, splenomegaly or histology was found through F_2 generation rats during 0.05 - 0.5% HMP and TPP or 0.1 - 1% c-triMP supplements (33).

Serology and Granulocytic Response

There was no change in serum calcium, inorganic phosphorus, alkaline phosphatase, protein and NPN or Hb and CO_2-combining capacity after high H_2PO_4 diets in humans (578). Neither total serum phosphorus nor pH was changed 1 hour post-injection of 60 drops H_3PO_4 in healthy or diabetic subjects (517). HMP and, to a slight extent, PP, but not oP, removed complement by combining with serum protein, an effect attributed to competition with protein anions (320). Orthophosphoric acid p.o. in hemorrhagic dogs stimulated erythropoeisis and normalization of rbc levels 1/3 faster than untreated controls. The left shift included a 100% increase in number of heterochromatic and nucleated rbc over controls. There was also a greater "post-hemorrhagic" leukocytosis, especially myelocytic and granulocytic (220). Bone marrow mitotic rates were significantly accelerated by sodium orthophosphate in stressed and unstressed rats (707). PP, oP, TPP, and HMP dietary supplementation (3-5%) in rats reduced rbc and Hb levels 10% below control animals, with PP and TPP having the greatest effect (355).

A modest eosinophilia and neutropenia were reported as concomitants of "phosphate nephritis" in dogs receiving 4mg/kg HMP, TPP, c-triMP and c-tetMP p.o. (33). ^{32}P recovery in serum after gavage delivery of $CaHPO_4$ and $Ca_3(PO_4)_2$ in rats was maximal at 24-48 hours, with subsequent proportional decreases (43). Serum oP was slight after i.v. administration of cyclic sodium tri- and tetrametaphosphate in rabbits and absent following i.p. delivery in rats. Serum oP was significantly higher after i.v. Ca-Na-HMP in both rat and rabbit (324). Label from $H_3{}^{32}PO_4$ given i.v. to rabbits reached a serum maximum 10-15 min post-treatment, with exponential decline similar to biliary extinction rates and a terminal 2 hour level significantly higher than that for bile (626). Serum ^{32}P peaked at 21 hours and total serum phosphorus peaked at 47 hours after pre-parturition $(NH_4)_2HPO_4$ rations and intraruminal labeling in ewes (723). Serum label after $CaH^{32}PO_4$ also peaked at 21 hours but total serum phosphorus was unaffected.

Renal Pathology

A high NaH_2PO_4 supplementation in rat rations for 1 month, followed by dietary calciferol and stock feedings, led to parenchymatous and tubular atrophy with cicatrix-type fibrosis and secondary glomerular-arteriolar hyaline degeneration, which resembled the histology of chronic nephritis (230). High levels of calcification were established following 3-5% oP, PP or TPP diet supplements in rats; HMP was without effect (355). The proximal segment of the broad ascending limb of Henle's loop was uniquely implicated as the site of base release and urine acidification: 7600 mg/kg NaH_2PO_4 in rats led to a localized pH-associated necrosis of littoral epithelium ("phosphate nephritis") (561). Tubular and interstitial atrophy, fibrosis, hyaline formation and calcification were construed as entirely secondary. A "phosphate necrosis" of the tubules was attributed to condensed polyphosphates, especially HMP and TPP, after 0.05%-0.5% dietary supplementation in rats (33). Tubular dilatation with interstitial and glomerular fibrosis and inter-tubular calcification was regarded as a separate syndrome ("chronic tubular nephropathy") consistent with 5% supplements. An acute renal pelvic inflammation was also characteristic of 2% polyphosphate diets after a 1 month feeding to rats. Weight loss, cardiomegaly, eosinophilia and neutropenia were associated with "phosphate nephritis" in dogs receiving 4mg/kg HMP, TPP, c-triMP and c-tetMP (33).

Acidosis or Hypocalcemia

Although total serum calcium was lowered in stressed and unstressed rats by Na_3PO_4 (707), the alkaline (Na_3HPO_4) has been thought to induce alkalosis with a reduction in ionized calcium without corresponding change in total serum or body calcium concentration. Conversely, the acid phosphate (NaH_2PO_4) has been thought to elicit acidosis and a relative increase in calcium ionization. Chemical equilibrium predicts that a relative acidosis and hypocalcemia are physiologically antagonistic and the degree of hypocalcemic insult would then depend on the amount of diffusible serum calcium sequestered.

Total inhibition of clotting by 0.1% HMP, and a slowed clotting reaction by lower concentrations were confirmed in graded dose-response studies. High renal calcification was found in rats on 3-5% oP, PP or TPP dietary supplements (355). $Ca_3(PO_4)_2$ was the most effective adsorbing surface for cholesterol accumulation in model systems simulating atheroma calcification (306). An 8% dietary sodium metaphosphate supplement study for 7 months in rats revealed gradual bone decalcification with metastatic calcium deposits, significant parathyroid hypertrophy and hyperplasia, inorganic phosphaturia and renal calcium deposition (primarily medullary) (792). Sodium pyrophosphate equivalent to 0.14, 0.21 or 1.2 mg P/kg s.c. injected simultaneously with calcifying agents s.c. reduced or prevented wheal calcification, hypercalcemia and hypercalcuria (except that from parathyroid extract). However, calcification of myocardium, aorta or proximal tubules was unaffected by 1.2 mg P (920). Using elongation of the Q-T interval, relative to cycle length R-R, in the EKG as an index of hypocalcemia, sodium TPP, HMP and PP i.v. were significantly toxic, while oP or c-tetMP had little effect and c-triMP led to Q-T contraction. A combination Ca-Na HMP salt i.v. was 3-7x more toxic than NaHMP alone, and led to an entirely different clinical and lethal outcome. NaHMP and TPP i.p. were negative even at high doses, while only PP i.p. was conspicuously hypocalcemic at LD levels (323). This result would tend to confirm that the yield of circulating PP from HMP or TPP hydrolysis is slight.

The condensed polyphosphates under review share roughly the same molar calcium-binding potential (per mol of P) in terms of precipitates

formed in supersaturated solutions of calcium. It would be expected that differences in hypocalcemic potential would depend on the stability of the calcium-phosphate complex rather than its stoichiometry and degree of cardiac arrest elicited i.v. by the linear polyphosphate LD's should then correlate specifically with their respective degrees of calcium sequestration, with the least trauma elicited by the most labile complexing agents, the cyclic polyphosphates and oP. The unexpectedly negligible hypocalcemia after i.p. treatment by the higher linear polyphosphates, HMP and TPP, could result from the countervailing systemic acidosis generated by rapid hydrolysis in the slow polymer release i.p. system. Accordingly, a metabolic acidosis was found to follow any and all parenteral administration; and parenteral host response curves, including systemic load data (688), suggested authentic drug-receptor activity.

The degree of acidotic insult would be expected to vary directly with the rate of acid release at physiological pH. Against the parenteral background, potential sources of alleged oral toxicity would be restricted only to massive oP formation and accumulation in the kidney. Based on some minimum "renal damage" (hemorrhagic exudate with cortical atrophy) from 1% PP supplements in rats (218) and a 100x safety margin factor, the PP renal clearance level would be 100x below 1% PP, or 0.01% in diet. This would approximate a daily PP intake of 6mg/kg for a 70kg human. Since p.o. polyphosphate urine excretion is equivalent to oP elimination and additional daily phosphate ingestion from all sources is unlikely to approach a level 100x higher, the rate of oP renal clearance would scarcely fall below the rate of polyphosphate hydrolysis to oP. Therefore, oral toxicity of orthophosphate must be dependent on transient accumulation of acid or alkaline phosphate on either side of the littoral gradients along the broad ascending limb of Henle's loop. But since normal renal phosphorus clearance is quantitative, at least within the FAO/WHO "unconditional" acceptance level in diet, only a gradient-dependent ephemeral accumulation of alkaline phosphate might be anticipated consequent to base exchange. Accordingly, a transient eosinophilia and neutropenia, cell correlates of alkalosis, were reported as systemic concomitants of "phosphate nephritis" after oral ingestion of both linear polyphosphates and cyclic oligomers.

CHEMICAL INFORMATION

PHOSPHORIC ACID

I. Nomenclature

A. Common names: phosphoric acid; orthophosphoric acid

B. Chemical name: phosphoric acid

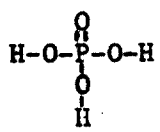
C. Trade name: none

D. CAS Reg. No. 7664382

II. Emperical Formula

H_3PO_4

III. Structural Formula



IV. Molecular Weight

98.00

V. Specifications

A. Food Chemicals Codex

limits of impurities

arsenic (as As) - 3 ppm

fluoride - 10 ppm

heavy metals (as Pb) 10 ppm

B. Chemical grade

1. Fisher Chemical Co.

assay - 85% min.

limits of impurities

chloride - 3 ppm

nitrate - 5 ppm

reducing substances - pass test

heavy metals - 10 ppm

sulfate - 1 ppm

volatile acids (as acetic) - 10 ppm

arsenic - 0.5 ppm

iron - 30 ppm

2. J. T. Baker Chemical Co.

   same as Fisher except

   insoluble matter (NH_4OH ppt) - 50 ppm

   sulfate - 30 ppm

   manganese - 0.5 ppm

   potassium - 50 ppm

   sodium - 250 ppm

3. M C & B Manufacturing Chemists

   same as Fisher except

   arsenic - 1 ppm

   manganese - 0.5 ppm

   sulfate - 30 ppm

VI. Description

A. General: unstable orthorhombic crystals, or, clear syrupy liquid; deliquescent

B. Physical Properties

m.p. 42.35°

solubility

miscible with H_2O and alcohol

C. Stability

stable in air

AMMONIUM PHOSPHATE, DIBASIC

I. Nomenclature

A. Common names: secondary diammonium phosphate; ammonium phosphate; diammonium hydrogen phosphate;

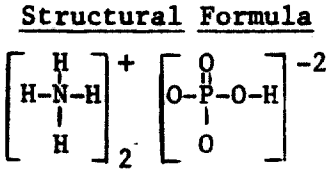
B. Chemical name: dibasic ammonium phosphate; phosphoric acid, diammonium salt

C. CAS Reg. No. 7783280

II. Empirical Formula

$(NH_4)_2HPO_4$

III. Structural Formula



IV. Molecular Weight

132.07

V. Specifications

A. Food Chemicals Codex

assay- not less then 96.0% and not more than 102.0% of $(NH_4)_2 HPO_4$

limits of impurities

arsenic (as As)- 3 ppm

fluoride - 10 ppm

heavy metals (as Pb)- 10 ppm

B. Chemical Grade

1. Fisher Chemical Co.

limits of impurities

insoluble matter - 50 ppm

nitrate - 30 ppm

chloride - 10 ppm

arsenic - 0.5 ppm

heavy metals - 10 ppm

iron - 10 ppm

NH_4OH ppt. - 50 ppm

potassium - 50 ppm

sodium - 50 ppm

sulfur compounds (as SO_4) - 40 ppm

pH of 0.2 M sol'n. -7.7-8.1

2. J. T. Baker Chemical Co.

same as Fisher Chemical Co. except

chloride - 5 ppm

3. M C & B Manufacturing Chemists

same as Fisher Chemical Co. except

alkali salts (as SO_4) - 1000 ppm

arsenic - 2 ppm

VI. Description

A. General: white, odorless crystals, crystalline powder, or granules

B. Physical Properties

m.p. - decomposes at 155°

solubility

H_2O - 575 gm/l at 10°

C. Stability

gradually loses about 8% NH_3 on exposure to air

AMMONIUM PHOSPHATE, MONOBASIC

I. Nomenclature

A. Common names: ammonium biphosphate; ammonium dihydrogen phosphate; primary ammonium phosphate; monoammonium phosphate;

B. Chemical name: monobasic ammonium phosphate, phosphoric acid, monoammonium salt

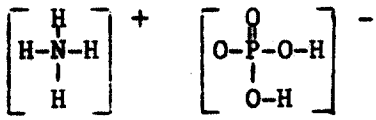
C. Trade name: none

D. CAS Reg. No. 7722761

II. Empirical Formula

$(NH_4)H_2PO_4$

III. Structural Formula



IV. Molecular Weight

115.03

V. Specifications

A. Food Chemicals Codex

assay - not less than 96.0% and not more than 102.0% of $(NH_4)H_2PO_4$

limits of impurities

arsenic (as As) - 3 ppm

fluoride - 10 ppm

heavy metals (as Pb) - 10 ppm

B. Chemical Grade

1. Fisher Chemical Co.

limits of impurities

insoluble matter - 50 ppm

NH_4OH ppt - 50 ppm

chloride - 5 ppm

heavy metals - 5 ppm

sulfur compounds - 50 ppm

potassium - 50 ppm - 50 ppm

nitrate - 10 ppm

iron - 10 ppm

sodium - 50 ppm

arsenic - 0.5 ppm

pH of 5% sol'n. at 25°C - 3.8-4.4

2. J. T. Baker Chemical Co.

   same as Fisher Chemical Co.

3. M C & B Manufacturing Chemists

   same as Fisher Chemical Co.

VI. Description

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

B. Physical Properties

m.p. 190°

solubility

H_2O - 227 gm/l at 0°

alcohol - slightly soluble

C. Stability

stable to air

AMMONIUM PHOSPHATE, TRIBASIC

I. Nomenclature

A. Common names: ammonium phosphate; ammonium phosphate tribasic;

B. Chemical names: tribasic ammonium phosphate; phosphoric acid, tri-ammonium salt

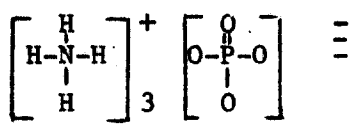
C. Trade name: none

D. CAS Reg. No. 10361656

II. Empirical Formula

$(NH_4)_3\ PO_4$

III. Structural Formula



IV. Molecular Weight

149.09

V. Specifications

A. Food Chemicals Codex

not listed

B. Chemical Grade

not listed

VI. Description

A. General: The trihydrate forms white prisms

B. Physical Properties

m.p. loses NH_3 upon heating

solubility

H_2O - 261 gm/l at 25°

C. Stability

loses NH_3

CALCIUM HEXAMETAPHOSPHATE

(all information found under the name calcium metaphosphate)

I. Nomenclature

A. Common names: calcium hexametaphosphate, calcium metaphosphate

B. Chemical names: calcium hexametaphosphate, calcium metaphosphate

C. Trade name: none

D. CAS Reg. No. 10102768 (calcium metaphosphate)

II. Empirical Formula

amorphous mixture; $CaO/P_2O_5 = 1$

III. Structural Formula

amorphous glass

IV. Molecular Weight

$CaO/P_2O_5 = 1$

V. Specifications

A. Food Chemicals Codex

not listed

B. Chemical Grade

not listed

VI. Description

A. General: amorphous glass

B. Physical Properties

m.p. - glass

solubility

H_2O - insoluble

C. Stability

stable in air

CALCIUM PHOSPHATE, DIBASIC

I. Nomenclature

A. Common names: calcium monohydrogen phosphate; secondary calcium phosphate; calcium biphosphate; calcium phosphate; calcium phosphate (dibasic); dicalcium phosphate; monocalcium acid phosphate; dicalcium orthophosphate

B. Chemical names: dibasic calcium phosphate; phosphoric acid, calcium salt (1:1)

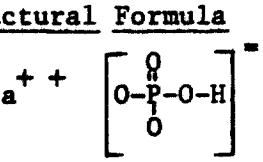
C. Trade name: none

D. CAS Reg. No. 7757939

II. Empirical Formula

$CaHPO_4$

III. Structural Formula



IV. Molecular Weight

136.06

V. Specifications

A. Food Chemicals Codex

assay - anhydrous $CaHPO_4$, not less than 39.0% and not more than 42% CaO; $CaHPO_4 \cdot 2H_2O$ (dihydrate), not less than 31.9% nor more than 33.5% CaO.

limits of impurities

arsenic (as As) - 3 ppm

fluoride - 50 ppm

heavy metals (as Pb) - 30 ppm

lead - 5 ppm

B. Chemical Grade

1. Fisher Chemical Co.

limits of impurities

insolubles in HCl - 100 ppm

carbonate - 200 ppm

nitrogen - 50 ppm

iron - 50 ppm

chloride - 20 ppm

sulfate - 100 ppm
barium - 100 ppm
arsenic - 1 ppm
heavy metals - 20 ppm
magnesium - 2000 ppm

2. J. T. Baker Chemical Co.
same as Fisher Chemical Co. except
assay - 98.0%
sulfite - 400 ppm
heavy metals - 30 ppm

3. M C & B Manufacturing Chemists
same as Fisher Chemical Co. except
sulfur - 200 ppm

VI. Description

A. General: It is anhydrous or contains two molecules of water of hydration. It occurs as a white, odorless, tasteless powder (Food Chemicals Codex).

B. Physical Properties
m.p.- 109°
solubility
H_2O - gm/l at 30°

C. Stability
stable in air

CALCIUM PHOSPHATE, MONOBASIC

I. Nomenclature

A. Common Names: monobasic calcium phosphate; phosphoric acid, calcium salt; calcium biphosphate; monocalcium phosphate; primary calcium phosphate; "calcium super phosphate"

B. Chemical names: monobasic calcium phosphate; phosphoric acid, calcium salt (2:1)

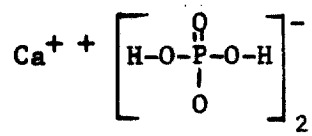
C. Trade name: none

D. CAS Reg. No. 7758238

II. Empirical Formula

$Ca(H_2PO_4)_2$

III. Structural Formula



IV. Molecular Weight

234.06

V. Specifications

A. Food Chemicals Codex

assay - anhydrous $Ca(H_2PO_4)_2$, not less than 23.5% and not more than 25.6% CaO; $Ca(H_2PO_4)_2 \cdot H_2O$ (monohydrate), not less than 22.2% and not more than 24.7% CaO.

limits of impurities -

arsenic (as As) - 3 ppm

fluoride - 25 ppm

heavy metals (as Pb) - 30 ppm

lead - 5 ppm

B. Chemical Grade

1. Fisher Chemical Co.

limits of impurities

insolubles in HCl - 100 ppm

chloride - 20 ppm

sulfate - 100 ppm

iron - 50 ppm

ammonia - 100 ppm

arsenic - 1 ppm

heavy metals - 20 ppm

2. J. T. Baker Chemical Co.

same as above except

assay - 99.0%

iron - 30 ppm

3. M C & B Manufacturing Chemists

same as Fisher Chemical Co. except

iron - 30 ppm

VI. Description

A. General: It occurs as white crystals or granules, or a granular powder (Food Chemicals Codex).

B. Physical properties

m.p. - decomposes at 200°

solubility -

H_2O- 18 gm/l at 30°

C. Stability

stable in air

CALCIUM PHOSPHATE, TRIBASIC

I. Nomenclature

A. Common names: tricalcium phosphate; tertiary calcium phosphate; bone ash; precipitated calcium phosphate

B. Chemical names: calcium phosphate, tribasic; phosphoric acid, calcium salt (2:3)

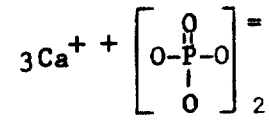
C. Trade name: none

D. CAS Reg. No. 7758874

II. Empirical Formula

$Ca_3(PO_4)_2$

III. Structural Formula



IV. Molecular Weight

310.20

V. Specifications

A. Food Chemicals Codex

assay - not less than the equivalent of 90.0% $Ca_3(PO_4)_2$

limits of impurities-

arsenic (as As) - 3 ppm

fluoride - 50 ppm

heavy metals (as Pb) - 30 ppm

lead - 5 ppm

B. Chemical Grade

1. Fisher Chemical Co.

limits of impurities

insolubles in HCl - 100 ppm

dibasic or free CaO - to pass test

carbonate (CO_3) - 300 ppm

chloride - 100 ppm

nitrate - 300 ppm

sulfide - 200 ppm

ammonia - 200 ppm

arsenic - 1 ppm

barium - 100 ppm

heavy metals - 20 ppm

iron - 50 ppm

magnesium - 300 ppm

soluble salts - 1.5%

2. J. T. Baker Chemical Co.

same as Fisher Chemical Co. except

chloride - 200 ppm

nitrogen - 500 ppm

no traces of CaO, barium, magnesium, soluble salts or ammonia.

3. M C & B Manufacturing Chemists

same as Fisher Chemical Co. except

no traces of CaO, barium, magnesium, or soluble salts, or ammonia.

chloride - 200 ppm

nitrogen - 500 ppm

VI. <u>Description</u>

A. General: Tribasic calcium phosphate consists of a variable mixture of calcium phosphates having the approximate composition of 10 $CaO \cdot 3P_2O_5 \cdot H_2O$. It occurs as a white, odorless, tasteless powder (Food Chemicals Codex).

B. Physical Properties

m.p. - 1670°

solubility - 0.02 gm/l in cold H_2O

C. Stability

stable in air

CALCIUM PYROPHOSPHATE

I. Nomenclature

A. Common names: calcium pyrophosphate; calcium diphosphate

B. Chemical name: calcium pyrophosphate; pyrophosphoric acid, calcium salt (1:2)

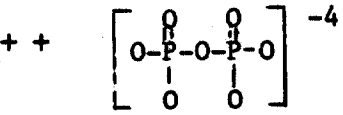
C. Trade name: none

D. CAS Reg. No. 7790763

II. Empirical Formula

$Ca_2P_2O_7$

III. Structural Formula



IV. Molecular Weight

254.10

V. Specifications

A. Food Chemicals Codex

assay - not less than 96.0% of $Ca_2P_2O_7$

limits of impurities

arsenic (as As) - 3 ppm

fluoride - 50 ppm

heavy metals (as Pb) - 30 ppm

lead - 5 ppm

B. Chemical Grade

not listed

VI. Description

A. General: a fine, white, odorless and tasteless powder; the crystals are polymorphous.

B. Physical Properties

m.p. 1230°

solubility

H_2O - insoluble

C. Stability

stable in air

POTASSIUM PHOSPHATE, DIBASIC

I. Nomenclature

A. Common names: dibasic potassium phosphate; dipotassium hydrogen phosphate; dipotassium monophosphate; dipotassium phosphate; dipotassium phosphate, dibasic; phosphoric acid, dipotassium salt; potassium phosphate; dikalium phosphate; dipotassium orthophosphate

B. Chemical names: dibasic potassium phosphate; phosphoric acid, dipotassium salt.

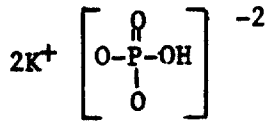
C. Trade name: none

D. CAS Reg. No. 7758114

II. Empirical Formula

K_2HPO_4

III. Structural Formula



IV. Molecular Weight

174.18

V. Specifications

A. Food Chemicals Codex

assay - not less than 98.0% K_2HPO_4 after drying

limits of impurities

arsenic (as As) - 3 ppm

fluoride - 10 ppm

heavy metals (as Pb) - 20 ppm

lead - 5 ppm

insoluble substances - not more than 0.2%

B. Chemical Grade

1. Fisher Chemical Co.

limits of impurities

insoluble matter - 100 ppm

arsenic - 2 ppm

carbonate - 2000 ppm

heavy metals (as Pb) - 5 ppm

iron - 10 ppm

loss on drying at 105°C - 2000 ppm
nitrogen compounds - 10 ppm
sodium - 300 ppm
sulfite - 50 ppm
pH of a 5% sol'n. at 25°C - 8.5 - 9.6

2. J. T. Baker Chemical Co.
   same as Fisher Chemical Co.
3. M C & B Manufacturing Chemists
   assay minimum - 99.0%
   arsenic - 1 ppm
   chloride - 30 ppm
   heavy metals (as Pb) - 5 ppm
   insolubles - 100 ppm
   iron - 10 ppm
   nitrogen (as N) - 10 ppm
   sodium - 500 ppm
   sulfite - 50 ppm

VI. Description

A. General: a colorless or white, granular salt which is deliquescent when exposed to moist air (Food Chemicals Codex).

B. Physical Properties
   m.p. decomposes
   solubility
   H_2O - 1670 gm/l at 20°
   alcohol - insoluble

C. Stability
   stable in air

POTASSIUM PHOSPHATE, MONOBASIC

I. Nomenclature

A. Common names: monopotassium phosphate; phosphoric acid, monopotassium salt; potassium acid phosphate; potassium biphosphate; potassium dihydrogen phosphate; monobasic potassium phosphate; Sorensen's potassium phosphate

B. Chemical names: monobasic potassium phosphate; phosphoric acid, dipotassium salt

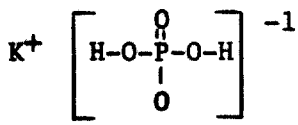
C. Trade name: Ultrex (J. T. Baker Chemical Co.)

D. CAS Reg. No. 7778770

II. Empirical Formula

KH_2PO_4

III. Structural Formula



IV. Molecular Weight

136.09

V. Specifications

A. Food Chemicals Codex

assay - not less than 98.0% of KH_2PO_4 after drying

limits of impurities

arsenic (as As) - 3 ppm

fluoride - 10 ppm

heavy metals (as Pb) - 20 ppm

lead - 5 ppm

insoluble substances - not more than 0.2%

B. Chemical Grade

1. Fisher Chemical Co.

limits of impurities

hydroxide ppt. - 100 ppm

sulfite - 30 ppm

loss on drying over H_2SO_4 - 0.2%

chloride - 10 ppm

nitrogen compounds (as N) - 10 ppm

heavy metals (as Pb) - 10 ppm

iron - 20 ppm

sodium - 50 ppm

pH of a 5% sol'n. at 25°C- 4.1 - 4.5

2. J. T. Baker Chemical Co.

same as Fisher Chemical Co.

3. M C & B Manufacturing Chemists

same as Fisher Co.

VI. Description

A. General: colorless crystals or white granular or crystalline powder (Food Chemicals Codex).

B. Physical Properties

m.p. 252.6°

solubility

H_2O - 330 gm/l at 25°C

alcohol - insoluble

C. Stability

stable in air

POTASSIUM PHOSPHATE, TRIBASIC

I. Nomenclature

A. Common names: potassium orthophosphate; tripotassium phosphate; potassium phosphate; tribasic potassium phosphate; phosphoric acid, tripotassium salt

B. Chemical names: tribasic potassium phosphate; phosphoric acid, tripotassium salt

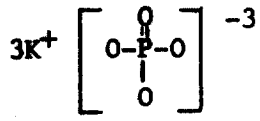
C. Trade name: none

D. CAS Reg. No. 7778532

II. Empirical Formula

K_3PO_4

III. Structural Formula



IV. Molecular Weight

212.28

V. Specifications

A. Food Chemicals Codex

assay - not less than 97.0% K_3PO_4

limits of impurities

arsenic (as As) - 3 ppm

fluoride - 10 ppm

heavy metals (as Pb) - 20 ppm

lead - 5 ppm

insoluble substances - not more than 0.2%

B. Chemical Grade

1. Fisher Chemical Co.

assay - 95% min.

limits of impurities

arsenic - 0

sulfite - 100 ppm

carbonate - trace

chloride - 50 ppm

heavy metals - 10 ppm

iron - 20 ppm

2. J. T. Baker Chemical Co.

excess alkali (as KOH) - 0.5%

insoluble matter - 100 ppm

chloride - 50 ppm

nitrogen compounds - 20 ppm

sulfite - 50 ppm

arsenic - 1 ppm

heavy metals - 10 ppm

iron - 10 ppm

3. M C & B Manufacturing Chemists

arsenic - 1 ppm

chloride - 50 ppm

heavy metals - 10 ppm

insoluble - 100 ppm

iron - 10 ppm

sulfite - 100 ppm

VI. <u>Description</u>

A. General: occurs as white, odorless, hygroscopic crystals or granules (Food Chemicals Codex).

B. Physical Properties

m.p. 1340°

solubility

H_2O - 900 gm/l at 20°

alcohol - insoluble

C. Stability

stable in air

SODIUM HEXAMETAPHOSPHATE

I. Nomenclature

A. Common names: sodium hexametaphosphate; sodium metaphosphate; metaphosphoric acid, hexasodium salt; Graham's salt

B. Chemical names: sodium hexametaphosphate; metaphosphoric acid, hexasodium salt

C. Trade names: Calgon, Micromet, Quadrafos

D. CAS Reg. No. 10124568

II. Empirical Formula

$Na(PO_3)_x$ approximately

III. Structural Formula

Amorphous sodium polyphosphate having an Na_2O/P_2O_5 mole ratio of about 1.1 (Food Chemicals Codex).

IV. Molecular Weight

a mixture of polymeric compounds

V. Specifications

A. Food Chemicals Codex

assay - not less than 66.5% nor more than 68.0% P_2O_5

limits of impurities

arsenic (as As) - 3 ppm

fluoride - 10 ppm

heavy metals (as Pb) - 10 ppm

insoluble substances - not more than 0.1%

B. Chemical Grade

1. J. T. Baker Chemical Co.

P_2O_5 content - 66.8 - 68.0%

2. M C and B Manufacturing Chemists

same as J. T. Baker Chemical Co.

VI. Description

A. General: It comes as a powder or colorless glass-like flakes.

B. Physical Properties

m.p. - a glass

C. Stability

stable in air

SODIUM METAPHOSPHATE

I. Nomenclature

A. Common names: sodium hexametaphosphate; sodium polyphosphate; sodium metaphosphate; Graham's salt

B. Chemical names: sodium metaphosphate; metaphosphoric acid, sodium salt

C. Trade name: metafos

D. CAS Reg. No. 10361032

II. Empirical Formula

$(NaPO_3)n$ $n>2$

III. Structural Formula

The amorphous and crystalline metaphosphates having Na_2O/P_2O_5 of 1.0 - 1.3. This group includes sodium hexametaphosphate.

IV. Molecular Weight

mixture

V. Specifications

A. Food Chemicals Codex

assay - 62.8% to 70.0 of P_2O_5, depending on class of ingredients

limits of impurities

arsenic (as As) - 3 ppm

fluoride - 10 ppm

heavy metals (as Pb) - 10 ppm

insoluble substances - not more than 0.1% for soluble forms

B. Chemical Grade

not listed; see sodium hexametaphosphate

VI. Description

A. General: occurs as colorless, glassy, transparent platelets or granules, or as a powder; often hygroscopic

B. Physical Properties

solubility

H_2O - usually very soluble, but insoluble forms exist

C. Stability

stable in air

SODIUM PHOSPHATE, DIBASIC

I. Nomenclature

A. Common names: disodium orthophosphate; phosphate of soda; secondary sodium phosphate; dibasic sodium phosphate; disodium hydrogen phosphate; disodium monohydrogen phosphate; disodium phosphate; phosphoric acid, disodium salt; soda phosphate; Sorensen's sodium phosphate;

B. Chemical names: phosphoric acid, disodium salt; dibasic sodium phosphate

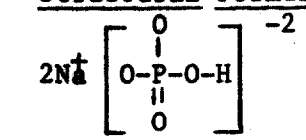
C. Trade name: none

D. CAS Reg. No. 7558794

II. Empirical Formula

Na_2HPO_4

III. Structural Formula



IV. Molecular Weight

141.96

V. Specifications

A. Food Chemicals Codex

assay - not less than 98.0% of Na_2HPO_4 after drying

limits of impurities

arsenic (as As) - 3 ppm

fluoride - 50 ppm

heavy metals (as Pb) - 10 ppm

insoluble substances - not more than 0.2%

B. Chemical Grade

1. Fisher Chemical Co.

limits of impurities

insoluble matter - 100 ppm

loss on drying at 105°C - 200 ppm

chloride - 20 ppm
nitrogen compounds - 20 ppm
sulfite - 50 ppm
heavy metals (as Pb) - 10 ppm
iron - 20 ppm
pH of 0.1 molar sol'n - 9.1 - 9.2 at 25°C

2. J. T. Baker Chemical Co.
   same as Fisher Chemical Co. except
   assay - 99%
   nitrogen compounds - 10 ppm
   iron - 10 ppm
3. M C & B Manufacturing Chemists
   same as Fisher Chemical Co.

VI. Description

A. General: Dibasic sodium phosphate is anhydrous or contains 2 molecules of water of hydration. It occurs as a white powder or crystalline solid. The anhydrous form is hygroscopic (Food Chemicals Codex).

B. Physical Properties
   m.p. (dihydrate) 60°
   solubility
   H_2O - 1 kg/l at 50°
   alcohol - insoluble

C. Stability
   stable in air

SODIUM PHOSPHATE, MONOBASIC

I. Nomenclature

A. Common names: monosodium dihydrogen phosphate; monosodium phosphate; phosphoric acid, monosodium salt; sodium acid phosphate; sodium biphosphate; sodium dihydrogen phosphate; monobasic sodium phosphate; monosodium orthophosphate; primary sodium phosphate

B. Chemical names: monobasic sodium phosphate; phosphoric acid, monosodium salt

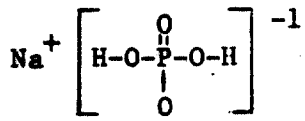
C. Trade name: none

D. CAS Reg. No. 7558807

II. Empirical Formula

NaH_2PO_4

III. Structural Formula



IV. Molecular Weight

119.98

V. Specifications

A. Food Chemicals Codex

assay - not less than 98.0% and not more than 103.0% of NaH_2PO_4, after drying

limits of impurities

arsenic (as As)- 3 ppm

fluoride - 50 ppm

heavy metals (as Pb) - 10 ppm

insoluble substances - not more than 0.2%

B. Chemical Grade

1. Fisher Chemical Co.

assay (as NaH_2PO_4) - 99% min.

limits of impurities

insolubles, Ca and NH_4OH ppt. - 100 ppm

chloride - 5 ppm

heavy metals (as Pb) - 10 ppm

nitrogen compounds (as N) - 10 ppm

sulfite - 30 ppm

arsenic - 0.5 ppm

iron - 10 ppm

pH of a 5% solution 4.1 - 4.5

2. J. T. Baker Chemical Co.

assay - 99.0%

same as Fisher Chemical Co.

VI. Description

A. General: It is anhydrous or contains one or two molecules of water of hydration. It is odorless and slightly hygroscopic. The anhydrous form is a white crystalline powder. The hydrated forms occur as

white or transparent crystals or granules (Food Chemicals Codex).

B. Physical Properties

m.p. (dihydrate) 60°

solubility

H_2O - 599 gm/l at 0°

ether - slightly soluble

C. Stability

stable in air

SODIUM PHOSPHATE, TRIBASIC

I. Nomenclature

A. Common names: sodium phosphate; sodium orthophosphate; tribasic sodium phosphate; phosphoric acid, trisodium salt; trisodium orthophosphate; trisodium phosphate; tertiary sodium phosphate

B. Chemical names: tribasic sodium phosphate; phosphoric acid, trisodium salt

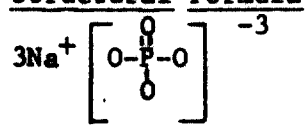
C. Trade name: none

D. CAS Reg. No. 7601549

II. Empirical Formula

Na_3PO_4

III. Structural Formula



IV. Molecular Weight

163.94

V. Specifications

A. Food Chemicals Codex

assay - anhydrous and monohydrate, not less than 97.0% of Na_3PO_4; dodecahydrate, not less than 92.0% Na_3PO_4, calculated on ignited basis

limits of impurities

arsenic (as As) - 3 ppm

fluoride - 50 ppm

heavy metals (as Pb) - 10 ppm

insoluble substances - not more than 0.2%

B. Chemical Grade

1. Fisher Chemical Co.

assay - 98% min.

limits of impurities

excess alkali (as NaOH) - not more than 2.5%

chloride - 10 ppm

iron - 10 ppm

nitrogen compounds (as N) - 10 ppm

sulfite - 100 ppm

arsenic - 5 ppm

heavy metals (as Pb) - 10 ppm

insoluble matter - 100 ppm

2. J. T. Baker Chemical Co.

assay - 98%

excess alkali (as NaOH) - 2.0%

insoluble matter

chloride - 10 ppm

nitrogen - 10 ppm

sulfite - 50 ppm

arsenic - 1 ppm

heavy metals - 10 ppm

iron - 10 ppm

3. M C & B Manufacturing Chemists

same as Fisher Chemical Co.

VI. Description

A. General: It is anhydrous or contains one or 12 molecules of water of hydration. It occurs as white, odorless crystals or granules or crystalline powder (Food Chemicals Codex).

B. Physical Properties

m.p. (dodecahydrate) 75°

solubility

H_2O - 88 gm/l in cold H_2O

alcohol - insoluble

C. Stability

stable in air

SODIUM ACID PYROPHOSPHATE

I. Nomenclature

A. Common names: disodium dihydrogen pyrophosphate; disodium pyrophosphate; sodium acid pyrophosphate; sodium pyrophosphate

B. Chemical names: pyrophosphoric acid, disodium salt, sodium acid pyrophosphate

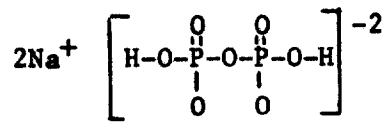
C. Trade name: none

D. CAS Reg. No. 7758169

II. Empirical Formula

$Na_2H_2P_2O_7$

III. Structural Formula



IV. Molecular Weight

221.94

V. Specifications

A. Food Chemicals Codex

assay - not less than 95% of $Na_2H_2P_2O_7$

limits of impurities

arsenic (as As) - 3 ppm

fluoride - 10 ppm

heavy metals (as Pb) - 20 ppm

lead - 5 ppm

insoluble substances - not more than 0.6%

B. Chemical Grade

not listed

VI. Description

A. General: white fused masses or free-flowing powder

B. Physical Properties

m.p. (hexahydrate) - decomposes at 220°

solubility

H_2O - 69 gm/l at 0°

C. Stability

stable in air

SODIUM PYROPHOSPHATE

I. Nomenclature

A. Common names: tetrasodium pyrophosphate; sodium pyrophosphate; tetrasodium diphosphate

B. Chemical names: sodium pyrophosphate; pyrophosphoric acid, tetrasodium salt

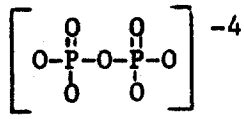
C. Trade name: Phosphotex; Tetron

D. CAS Reg. No. 7722885

II. Empirical Formula

$Na_4P_2O_7$

III. Structural Formula



IV. Molecular Weight

265.90

V. Specifications

A. Food Chemicals Codex

assay - not less than 95.0% of $Na_4P_2O_7$

limits of impurities

arsenic (as As) - 3 ppm

fluoride - 50 ppm

heavy metals (as Pb) - 10 ppm

insoluble substances - not more than 0.2%

B. Chemical Grade

1. Fisher Chemical Co.

limits of impurities

insolubles, calcium and NH_4OH ppt - 100 ppm

carbonate - to pass test

chloride - 10 ppm

nitrogen - 20 ppm

orthophosphate - 2000 ppm

sulfite - 100 ppm

arsenic - 1 ppm

heavy metals - 10 ppm

iron - 10 ppm

2. J. T. Baker Chemical Co.

   assay ($Na_4P_2O_7 \cdot 10H_2O$) - 99.0 - 101.0%

   insoluble matter - 100 ppm

   pH of 5% solution at 25°C - 9.5 - 10.5

   chloride - 20 ppm

   nitrogen - 10 ppm

   sulfite - 50 ppm

   arsenic - 5 ppm

   heavy metals - 10 ppm

   iron - 10 ppm

3. M C and B Manufacturing Chemists

   same as J. T. Baker Chemical Co.

VI. Description

A. General: either anhydrous or containing 10 molecules of water of hydration; It occurs as a white, crystaline or granular powder (Food Chemicals Codex).

B. Physical Properties

m.p. (anhydrous) 880°

solubility

H_2O - 31.6 gm/l at 0°

alcohol - insoluble

C. Stability

a) the decahydrate effloresces slightly in dry air

b) stable in air

SODIUM TRIPOLYPHOSPHATE

I. Nomenclature

A. Common names: pentasodium triphosphate; sodium triphosphate; sodium tripolyphosphate

B. Chemical names: triphosphoric acid, pentasodium salt; sodium tripolyphosphate

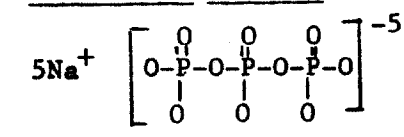
C. Trade name: none

D. CAS Reg. No. 7758294

II. Empirical Formula

$Na_5P_3O_{10}$

III. Structural Formula



IV. Molecular Weight

367.86

V. Specifications

A. Food Chemicals Codex

assay - not less than 85% $Na_5P_3O_{10}$

limits of impurities

arsenic (as As) - 3 ppm

fluoride - 50 ppm

heavy metals (as Pb) - 10 ppm

lead - 5 ppm

insoluble substances - not more than 0.1%

B. Chemical Grade

grade not listed

VI. Description

A. General: It is anhydrous or contains 6 molecules of water of hydration. It occurs as white, slightly hygroscopic granules, or as a powder.

B. Physical Properties

m.p. - not available

solubility

H_2O - 145 gm/l at 25°

C. Stability

With prolonged heating of solutions it tends to revert to orthophosphate

Analytical Methods

1. Separation of phosphate, pyrophosphate (PP) and condensed phosphates by chromatography and electrophoresis

(a) Ref. 918

Meat is extracted with H_2O. Orthophosphate and PP are separated by paper chromatography using isopropyl alcohol-H_2O-HCOOH solvent and the spots are developed with ammonium molybdate and vanadyl chloride. To quantitate PP an undeveloped spot is digested in H_2SO_4 and H_2O_2 and analyzed colorimetrically after ammonium molybdate treatment.

In foods which contain 0.4% PP 0.12μg can be determined. Relative error is 5%.

(b) Ref. 287

Phosphate mixtures are analyzed by paper chromatography using several solvents. Orthophosphate, PP, tripolyphosphate (TPP), trimeta- and tetrametaphosphate are completely separated and higher polymers are partially isolated.

Electrophoresis at 110 or 220v and 0.4-1.2mA in borate buffer at pH 9.2 isolates orthophosphate, PP and TPP but other condensed phosphates are not separated.

Both methods are made quantitative by digesting a spot in H_2SO_4, adding ascorbate and ammonium molybdate, and analyzing the solution colorimetrically. A 2-3% error is expected with both methods for 1-10μg samples.

2. Quantitative analysis of orthophosphate

In most cases condensed phosphates would be converted to orthophosphate during the determination.

A. Chromatography

(a) Ref. 131

Apple juice: The organic and inorganic acids are concentrated on a silicic acid column and phosphoric acid is separated from the mixture by paper chromatography, using several solvents and developers.

(b) Ref. 448

The color of orthophosphate chromatogram spots are enhanced by complexing the initial phosphomolybdate precipitate to basic dyes.

B. Colorimetry

(a) Ref. 78

Stock food calcium phosphate is separated into the monobasic, dibasic and tribasic components. $Ca(H_2PO_4)_2$ is selectively dissolved from a weighed sample in H_2O. The undissolved residue is heated at 600° and the $CaHPO_4$ fraction is dissolved in 20% sodium EDTA, leaving $Ca_3(PO_4)_2$ solid, which is dissolved in HNO_3. The concentrations of the three solutions are determined colorimetrically at 395 mμ using ammonium molybdate and ammonium vanadate. An accuracy of ± 2% is obtained.

(b) AOAC, 1970 p. 135

Plants and plant parts: A $Mg(NO_3)_2$ solution is added to a weighed sample and it is dried and ashed. The ash is dissolved in HCl and molybdovanadate reagent is added. The concentration is determined colorimetrically.

(c) Ref. 68

Yeast analysis: The sample is ashed in the presence of K_2CO_3 and H_2O_2. The ash is dissolved and neutralized with H_2SO_4. A few drops of sulfo-molybdate blue reagent, with Cu particles as a reducing agent, are added to a boiling solution. The absorption is measured (max. 650 mμ). Ca does not interfere and an accuracy of 5% can be obtained on solutions containing 2ppm.

(d) Ref. 1000

Egg yolk and fatty tissue: After converting an aqueous solution of phosphate to phosphomolybdate the latter was extracted with butyl acetate and the absorbance at 310 mμ was determined. From 0.3-5μg were determined.

C. Gasometric microanalysis

(a) Ref. 478

Whole blood, plasma or urine are analyzed. Trichloroacetic acid (10%) is added to the sample and the solution is filtered. The filtrate is neutralized and free phosphate is precipitated with Embden's strychnine molybdate reagent. CO_2, from the strych-

nine attached to the precipitate, is measured after combustion. Total phosphate may be determined by digesting the sample with H_2SO_4 before precipitation. For 10-20 μg P samples the error is ± 0.5%.

D. Gravimetry

(a) Ref. 289

A phosphate sample is dissolved in HNO_3 and precipitated with ammonium molybdate and NH_4NO_3. The precipitate is filtered, washed and heated at ca. 350°, leaving $(NH_4)_3 PO_4(MoO_3)_{12}$.

If the author's procedure is followed, which minimizes interferences from precipitates of ammonium dodecamolybdate and ammonium nonamolybdate, an accuracy of 0.2% and a precision of 0.1% can be obtained.

(b) AOAC, 1970 pp. 389, 11, 142

Meat samples are weighed and digested in HNO_3 and HCl. The solution is made slightly acid and NH_4NO_3 and ammonium molybdate precipitate the orthophosphate. The precipitate is washed and dissolved in NH_4OH. A magnesia mixture is added and the new precipitate is washed, ignited and weighed as $Mg_2P_2O_7$.

Titration

(a) Ref. 902

Tomato and egg product microanalysis: Minced food is extracted with alcohol; magnesium acetate is added to the extract and it is concentrated and ashed. HNO_3 dissolves the ash and ammonium molybdate with NH_4NO_3 precipitate phosphate. This precipitate is washed, dissolved in a known volume of standard NaOH and the solution is titrated.

(b) AOAC, 1970 pp. 286.

Eggs: To a weighed sample add a Na_2CO_3 solution. Evaporate and ash. Dissolve the ash in HCl and add ammonium molybdate with NH_4NO_3 to precipitate phosphate. Wash, add a measured volume of standard NaOH. Titrate to a phenolphthalein end point.

(c) AOAC, 1970 pp. 135, 13, 14

Plants: Add $Mg(NO_3)_2$ solution to weighed sample. Evaporate and ash the sample. Continue as in part (b).

(d) AOAC, 1970 pp. 520, 160, 13, 14

Malt beverages and vinegar: Add Ca acetate solution to the sample. Dry and ash. Continue as in (b).

(e) Ref. 744

Aqueous solutions of orthophosphate are titrated to the first equivalence point of phosphoric acid. $Ce(NO_3)_3$ precipitates $CePO_4$ and the remaining solution is then titrated back to the first equivalence point. Precision is 0.3% and Ca does not interfere.

(f) Ref. 471

H_3PO_4 and/or phosphate salt mixtures are titrated with NaOH. The course of neutralization is followed conductometrically. The mean error is ± 2%.

Occurrence

A. Plants: Phosphate is a minor constituent of all plants, from unicellular to the most complex. The phosphate fraction (free plus bound) of some plant parts is listed (Ref. 48).

Approximate total P content

	ppm
banana	1,250
mushroom	8,000
cocoa	8,400
orange juice	212

B. Animals: It is also an essential minor component of all animal tissue, from the simplest species to the most evolved. Phosphate is also a major constituent of mammalian bone (as calcium phosphates). Approximate phosphate contents given below are taken from ref. 48 and *Textbook of Medical Physiology*, Arthur C. Guyton, W. B. Saunders Co., 1971, p. 858, 931.

Approximate P contents

whole egg	2,900 ppm
fresh milk	1,230 ppm
adult man (total)	9.6 gm/kg
human serum (inorganic P)	3.5 - 4 mg/100 ml

C. Synthetics: Condensed phosphates are prepared by heating the corresponding orthophosphate salt, to drive off H_2O.

D. Natural Sources: Phosphate deposits occur mostly as the insoluble calcium salt, notably fluoroapatite. The amount of soluble phosphate is low because it is precipitated by Ca^{+2} at neutral and alkaline pH.

E. Uses as Food additive

(1) Agent to adjust pH (carbonated beverages)

(2) Acid reacting material (baking powder)

(3) Emulsifying, gelling, stabilizing and thickening agents (process chese, cream cheese)

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

(5) Anticaking and firming agent (salt, garlic and onion salt, canned

vegetables)

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

BIOLOGICAL DATA

I. Acute toxicity

1. Ref. 1012 (condensed phosphates)

Animal	Sex/No.	Route	Dose (mg/kg)	Measurement
		Na hexametaphosphate		
Mouse	?/?	p.o.	7250	LD_{50}
		s.c.	1300	"
		i.v.	62	"
		Na tripolyphosphate		
Mouse	?/?	p.o.	3210	LD_{50}
		s.c.	900	"
		i.v.	71	"
		sodium acid pyrophosphate ($Na_2H_2P_2O_7$)		
Mouse	?/?	p.o.	2650	"
		s.c.	480	"
		i.v.	59	"
		hexasodium tetrapolyphosphate		
Mouse	?/?	p.o.	3920	LD_{50}
		s.c.	875	"
		i.v.	----	----
		tetrasodium pyrophosphate		
Mouse	?/?	p.o.	2980	LD_{50}
		s.c.	400	"
		i.v.	69	"
		cyclic sodium trimetaphosphate		
Mouse	?/?	p.o.	10300	"
		s.c.	5940	"
		i.v.	1165	"

Host response curves after p.o. delivery simulated a non-specific osmotic "salt-effect". Disodium acid pyrophosphate, hexasodium tetrapolyphosphate, sodium hexametaphosphate and sodium tripolyphosphate effects i.v. or s.c. suggested pharmacological receptor activity.

2. Ref. 323 (condensed phosphates)

Animal	Sex/No.	Route	Dose(mg P/kg)	Measurement
		sodium pyrophosphate		
Rat	F/68	i.p.	59	LD_{50}
		sodium tripolyphosphate		
"	F/24	i.p.	135	LD_{50}
		sodium hexametaphosphate		
"	M/29	i.p.	192	LD_{50}

	sodium tetrametaphosphate			
"	M/113	i.p.	741	LD_{50}
	sodium orthophosphate (NaH_2PO_4-Na_2HPO_4)			
"	M/70		326	LD_{50}
	sodium trimetaphosphate			
"	F/57	i.p.	1130	LD_{50}
	calcium hexametaphosphate			
"	M/?	i.p.	576	LD_{50}

Intraperitoneal toxicity of sodium hexametaphosphate was manifest by immediate behavioral agitation even at sublethal doses (100 mg P/kg) with subsequent significant weight loss. At LD levels most deaths occurred 1-4 hours post-treatment. The LD_{50} of calcium hexametaphosphate (mg P/kg) was minimally 3x that of the sodium homolog, with some fatalities occurring only after several days of large doses of the calcium salt.

3. Ref. 218 (sodium pyrophosphate)

Sodium pyrophosphate lethal dose

Species	Sex/no.	route	dose	measurement
Rat	M/6	i.p.	1000 mg/kg	LD_{100}*
"	"	"	631"	"
"	"	"	398"	"
"	"	"	251"	LD_{67}*

*Refers to the number of animals dying in 16 h.

Doses of 25, 40 and 63 mg/kg sodium pyrophosphate were also given i.p. to 2 male Birmingham-Wistar rats. All were without effect, excepting some evidence of weakness and reduced mobility at the higher level. Six rats receiving 100 mg/kg i.p. showed a higher degree of weakness, with gasping respirations, but no fatalities; a similar, but more exacerbated, response obtained for 6 rats receiving 159 mg/kg i.p., with no deaths. A median LD_{50} was calculated to be 233 mg/kg (range 192-282).

Rats receiving either 631 or 1000 mg/kg NaPP became increasingly moribund (asthenic and anesthetic), with death 2 h post-treatment; there was no evidence of corneal reflex loss but respiration became slower and shallower. Autopsy revealed serous inflammation of the small gut.

In an independent study 1600 mg/kg NaPP was given p.o. to 10 rats. Sacrifice after 10 days revealed no abnormality. Intestinal motility was unimpaired in 2 rats receiving a 2% soln of 400 mg/kg Na_2HPO_4 and sacrificed 1 h post-treatment, while NaPP at this level elicited mucosal hyperemia and reduced motility. Serum calcium fell from 9.4 to 5.1 mg % in rats receiving 800 mg/kg NaPP, while the same dose of sodium ortho-

phosphate had little effect. Intradermal injection caused a necrotic outcome after 1% NaPP and slight ecchymoses after 4% Na_2HPO_4; NaPP was 8x as toxic intradermally.

4. Ref. 299 (NaH_2PO_4)

Substance	Species	Sex/no.	route	dose	measurement
NaH_2PO_4	rabbit	?/9	i.v.	1205 mg/kg	MELD*

*minimum eventually lethal dose

Rabbits 1300-1710 g in weight were infused i.v. with NaH_2PO_4 at the 39 mg $Na_2HPO_4 \cdot 2H_2O$/kg/min. Animals 1-5 received total doses of 1112 - 1366 mg/kg over the course of 58 - 77 min; all survived. Animals 6-9 received from 1483 - 1600 mg/kg over 85-92 min; survival time ranged from 67 1/2 to 1 1/2 hours post-termination of injection.

With non-lethal doses the animals were tranquil until the onset of muscular tremors followed by tonic-clonic convulsions, particularly in the hind quarters. Respiratory rate was always increased, diarrhea occurred and the animal frequently voided larged quantities of acidic urine. Heart rate was not modified notably.

With lethal doses the symptoms were similar, when death occurred rapidly, convulsive dyspnea was followed by cardiac arrest. Those that survived longer post-injection showed increasing depression and finally collapse and coma.

Histology showed considerable congestion of the organs, particularly intense in the kidney, with small parenchymal hemorrhages. The observed symptomatology and anatomical lesions, particularly the renal excretory burden indicates acidosis as the prime cause of death. Decalcification is also implicated as a secondary factor, especially since simultaneous administration of calcium chloride was found to prolong survival time.

5. Ref. S1 (NaH_2PO_4)

Diarrhea occurred in albino rats, guinea pigs and rabbits when doses of 250 mg/kg or greater of NaH_2PO_4 were administered by stomach tube. The LD_{50} of sodium acid phosphate, administered intramuscularly to albino rats, was found to be 250 mg NaH_2PO_4/kg body weight, when administered to to 44 animals.

6. Ref. 747 (orthophosphate)

Food was withdrawn the third day after adrenalectomy in 170-210 g male Sprague-Dawley rats with i.p. phosphate (pH 7.4) 24 hours later. All rats receiving 57 or 78 mg P/kg died within 15 hours after injection, on the average. However, all adrenalectomized rats that also received cortical extract (dose ?) survived a dose of 150 mg P/kg phosphate for at least 5-7 days. Without adrenalectomy it required 300 mg P/kg to kill all rats within an average of 4.4 hours.

7. Ref. 688 (Na_2HPO_4)

Substance	species	route	dose	measurement
Na_2HPO_4	rabbit	i.v.	486 mg/kg	MELD**
"	"	"	651 mg/kg*	MTD

**minimum eventually lethal dose

***miminum tetanizing dose

With the MELD, a marked cachexia and nephrosis occurs; death follows in a few days. Large doses elicit tetany and death is attributed to hematological and tissue changes (especially neural).

A discussion in ref. (299) implicates the affinity of the dibasic phosphate for calcium in the tetanic response.

II. Short-term studies

1. Ref. 355 (orthophosphate and condensed phosphates)

For 6 months 50-60 gm male and female rats were fed a diet supplemented with 3 or 5% phosphate (calculated as P_2O_5) in the form of orthophosphate, pyrophosphate, tripolyphosphate and Graham's salt (HMP). Body weights, measured every three weeks, were lower than controls in groups receiving a 3% supplement (but not 1.8% supplement) of any form of phosphate and this effect was more pronounced in groups fed a 5% supplement. Erythrocyte count and hemoglobin level were within 10% of controls on all types of supplement and an increase in average weights of the liver, kidney and especially the spleen was noticeable at the end of the experiment without recognizable connection with kind and concentration of phosphate. P, Fe, Cu and Ca content of completely ashed animals were within control limits, except for high levels of Ca and Fe in females fed 5% pyrophosphate and a high level of Cu in females fed 5% Graham's salt. Calcification was pronounced, especially in the kidneys, in groups fed 3 or 5% supplements, although it was less pronounced when Graham's salt was administered than others.

2. Ref. 33 (condensed phosphates)

Male weanling rats were maintained for 1 month on stock ration with various condensed phosphate additives. Growth retardation followed all 10% supplements of the sodium salts of hexametaphosphate, tripolyphosphate, and cyclic trimeta- and tetrametaphosphate (5 animals per group). Relative splenomegaly was found for 10% TPP, HMP and cyclic tetrametaphosphate, in decreasing order, at sacrifice on days 3, 7, 15 and 28. The kidneys were pale and swollen after 10% HMP. Renal tubular necrosis ("phosphate nephritis") was remarkable after 10% HMP and prominent with 10% cyclic trimeta- or tetrametaphosphate and 5% sodium orthophosphate. The trimetaphosphate and oP effects may have been transient. Acute pelvic inflammation was found on day 28 in some rats for all 2% diets, with tubular lesions ascribed to condensed phosphates in 3 rats; 0.2% had no effect.

Four young adult dogs received rations supplying either 0.1 mg/kg or graded 1-4 mg/kg sodium hexameta-, tripoly-, trimeta- and tetrametaphosphate daily for 1 month. At 4 mg/kg levels there was evidence of weight loss, eosinophilia and neutropenia, cardiomegaly, renal tubule injury resembling rat phosphate nephritis and left heart hypertrophy.

3. Ref. 218 (sodium pyrophosphate and orthophosphate)

Five groups of Birmingham-Wistar rats, including 20 males and 20 females each, received 1, 2.5 or 5% sodium pyrophosphate (PP) or 5% Na_2HPO_4 (oP) diets for 16 weeks. Mean body weight gain and cumulative food intake was lower for both sexes on 5% PP, but both values were statistically insignificant by covariance assay. Urinary pH was significantly less acid after 5% PP or oP (7.5) than that after lower PP rations (6.7), but urinary sodium, potassium or ammonium varied insignificantly. Approximately 75-80% of the phosphorus ingested in 5% PP diets was excreted in urine and 15-20% in feces, which values were compatible with the fate of oP. PP was never detected in feces or urine. Renal concentration tests found reduced function after 2 1/2% PP, 5% PP and 5% oP, while liver function (BSP) was not significantly altered. Erythrocyte counts and hemoglobin levels remained relatively unchanged. Leukocyte levels fluctuated vicariously, with both granulocyte and agranulocyte maxima reached after 5% PP for males. Elevations in relative heart, stomach, kidney and testis weight were significant for males, and heart, stomach, gut and kidney for females, on 5% PP; there was no evidence of splenomegaly or hepatomegaly. The only significant increase after 5% oP was renal enlargement in both sexes. Gastric hypertrophy and hemorrhage at the cardiac and pyloric borders were found in males after 5% PP and females after 2.5 or 5% PP. Macroscopic renal calcification with pitting was found for 5% PP and oP in males and 2.5-5% PP and 5% oP in females. Orthophosphate was not implicated in gastrointestinal inflammation. Cortical atrophy and hyaline degeneration were found for 1 and 2.5% PP, but not for 5% PP nor oP. Medullary calcification and necrosis, chronic inflammation and tubular casts were chiefly found at the 2.5 - 5% PP and 5% oP levels, while exudative hemorrhage and evidence of "renal damage" were characteristically equivalent for all PP and oP supplements.

4. Ref. 230 (NaH_2PO_4)

<u>Method</u>

species: rat

sex/no: both/300

duration of study: up to 21 months

vehicle: in diet (bread and potatoes)

dose schedule: 5% of diet for 20-30 days with concurrent p.o. administration of 10-40,000 units of calciferol, followed by a "normal" mixed diet.

Observations

Symptomatology: none noted

Gross pathology: chronic, progressive renal lesions of a fibrotic nature

Histopathology: very similar to chronic human nephritis; see discussion

Discussion

Animals succumbed or were sacrificed from the sixth to the six hundredth day of the study. The only tissue investigated was the kidney, the chief lesion being a parenchymatous degeneration with tubular atrophy and residual scar-like fibrosis. Severe, secondary changes included glomerular and arteriolar hyaline degeneration. The scar-like fibroses were shown to contain no new tissue and the collagen arising in the latter stages seemed to be derived from reticular fiber transformation alone. In the early stages, especially in those animals examined during phosphate dosage, edema was frequently apparent near the necrosing tubules and might be related to a secondary process involving interstitial hypertrophy.

5. Ref. 943 ($(NH_4)_2HPO_4$ and Na_2HPO_4)

Young cattle were fed a diet which included either $(NH_4)_2HPO_4$ or Na_2HPO_4 as phosphate supplement (approximately 6.3 gm phosphorus/kg feed), for 6-7 months. The diets contributed to a good weight increase. The animals suffered no illness attributable to the phosphate and no abnormal changes were found in the carcasses or internal organs upon examination after slaughter.

6. Ref. S2 ($Na(NH_4)_2PO_4$ and $(NH_4)_2HPO_4$)

Twice daily for 5-14 months female dogs received 0.1 - 0.2 g/kg $Na(NH_4)_2PO_4$ or 0.3 - 0.7 g/kg $(NH_4)_2HPO_4$ in 100-150 ml drinking H_2O, in gradually increasing doses. The average weight of the parathyroid increased 2.5x and 3.3x above controls with $Na(NH_4)_2PO_4$ and $(NH_4)_2HPO_4$ treatment, respectively. Extensive hypertrophy was also produced by other acidotic compounds.

7. Ref. 561 (NaH_2PO_4)

Method

Species: Albino rat

strain: Wistar

duration of study: A. 12 h - 3 days; B. 7 h - 42 days

vehicle: A. not given; B. milk

dose schedule: A. 2400 mg/kg/day; B. 7600 mg/kg/day (based on a mean rat weight of 250 g)

route of administration: A. i.p.; B. p.o. (in milk)

number of animals: A. 8; B. 15

The rats on schedule A. showed signs of suffocation and uneasiness at various post-injection intervals; on sacrifice, 5 gave evidence of incipient renal damage. The rats on schedule B showed no signs of behavioral difficulty with a milk mixture containing 3.3% NaH_2PO_4. The early histopathology of phosphate nephritis began 12 h post-treatment and was similar for both experimental groups. The pattern included initial nuclear pyknosis, coagulative necrosis and lysis of cells in a 200-400 μ proximal segment of the broad ascending limb of Henle's loop. Subsequent changes were: increasing degeneration of tubular epithelium, dilatation and hyalinization of the narrow limb of Henle's loop, calcification of necrotic areas of the broad limb, tubule dilatation, macrophage invasion and hyperplasia of both surviving epithelium and juxtaposed interstitial cells - all secondary to the original broad limb necrosis. This "phosphate nephritis" was attributed to excess acid phosphate accumulation at the inferred site of urine acidification, whose concentration elicited a pH-dependent epithelial-cell necrosis.

8. Ref. 270 (condensed phosphates)

Sprague-Dawley rats received 5 ml each P^{32} labeled tetrasodium diphosphate, pentasodium triphosphate and sodium polyphosphate (64% P_2O_5) by gavage. Sacrifice at various intervals and serological assay were run concurrently. Orthophosphate and adenosine monophosphate were identified chromatographically in deproteinated whole blood. The variation in serological label was a function of gastrointestinal transit interval as orthophosphate, which was resorbed and included in intermediate metabolism. Failure of polyphosphate resorption, due to membrane impermeability of condensed polyphosphates, was also confirmed. It is concluded that p.o.

polyphosphate effects are equivalent only to the monophosphate effect.

9. Ref. 794 (orthophosphate and metaphosphate)

Various tissue distributions of different phosphates (72 mg/kg) administered by gavage in young Wistar rats is shown in the following table after sacrifice at 4 or 24 hr:

TABLE 1

SUMMARY OF RADIOPHOSPHORUS CONTENT OF BLOOD, SALIVA, FEMUR, AND ENAMEL AS FURNISHED BY VARIOUS SOURCES OF PHOSPHATE AFTER 4- AND 24-HOUR TIME PERIOD AFTER INTUBATION (SERIES I)

		4-Hour Data			
Group	Phosphate Regimen	Blood (cpm/ml)	Saliva (cpm/ml)	Femur (cpm/mg ash)	Enamel (cpm/hemijaw)
A	None (cont.)	2.83* ± 0.57†	3.39 ± 0.89	0.40 ± 1.98	0.54 ± 0.52
B	NaH_2PO_4	6,675 ± 493‡	598.3 ± 25.3	330.0 ± 24.0	398.3 ± 24.5
C	Na_2HPO_4	5,959 ± 300	556.0 ± 11.0	312.6 ± 12.6	389.5 ± 42.8
D	KH_2PO_4	6,298 ± 400	399.0 ± 45.6	376.1 ± 14.2	413.9 ± 43.6
E	K_2HPO_4	6,215 ± 478	398.0 ± 37.9	340.1 ± 37.0	228.7 ± 20.6
F	$NH_4H_2PO_4$	8,686 ± 599	610.4 ± 77.4	386.1 ± 25.0	270.4 ± 15.2
G	$(NH_4)_2HPO_4$	7,297 ± 67	408.9 ± 55.4	333.6 ± 13.9	279.2 ± 11.7
H	$Ca(H_2PO_4)$	7,198 ± 560	405.6 ± 19.3	380.6 ± 23.7	401.2 ± 39.3
I	$CaHPO_4$	7,320 ± 834	379.4 ± 18.4	341.6 ± 16.2	291.4 ± 9.7
J	m-HPO_3	7,289 ± 376	531.1 ± 66.3	371.7 ± 15.2	296.2 ± 45.7

		24-Hour Data			
Group	Phosphate Regimen	Blood (cpm/ml)	Saliva (cpm/ml)	Femur (cpm/mg ash)	Enamel (cpm/hemijaw)
A	None (cont.)	2.32 ± 1.75	6.39 ± 1.56	0.75 ± 1.39	4.34 ± 0.27
B	NaH_2PO_4	4,439 ± 217	172.5 ± 14.3	389.5 ± 24.2	485.5 ± 14.8
C	Na_2HPO_4	3,196 ± 68	118.1 ± 30.3	288.1 ± 25.8	343.1 ± 60.1
D	KH_2PO_4	4,285 ± 107	118.8 ± 6.5	403.3 ± 16.2	385.3 ± 31.4
E	K_2HPO_4	3,723 ± 204	133.6 ± 10.2	362.2 ± 15.4	313.9 ± 27.8
F	$NH_4H_2PO_4$	3,881 ± 150	161.7 ± 17.3	455.5 ± 23.0	302.3 ± 37.8
G	$(NH_4)_2HPO_4$	4,045 ± 88	161.8 ± 22.4	384.4 ± 20.8	268.4 ± 20.8
H	$Ca(H_2PO_4)_2$	4,116 ± 144	123.2 ± 7.5	434.5 ± 20.4	350.1 ± 52.2
I	$CaHPO_4$	3,786 ± 263	132.3 ± 11.9	367.1 ± 20.0	257.1 ± 22.8
J	m-HPO_3	3,342 ± 247	115.2 ± 32.9	364.0 ± 31.9	239.8 ± 40.1

* Net value after correcting for background.
† Standard error of the mean.
‡ Net cpm value after correcting for background and decay loss.

"m-HPO_3" designates metaphosphate

Various tissue distributions of acid and alkaline phosphate, each given as 1% in a combined diet supplement for 2 weeks in rats before receiving 50 mg/kg of each separately by gavage, with sacrifice at times is indicated in the table on the following page:

TABLE 2

RADIOPHOSPHORUS CONTENT OF VARIOUS TISSUES IN RAT AS A FUNCTION OF TIME AFTER SINGLE ORAL INTUBATION OF ^{32}P PROVIDED AS NaH_2PO_4, Na_2HPO_4, OR A COMBINATION OF NaH_2PO_4 AND Na_2HPO_4 (SERIES II)

Group	Source of Phosphate	Time (hrs)	Sound Enamel (cpm)	Saliva (cpm/ml)	Blood (cpm/ml)	Ashed Molars (cpm/mg ash)	Femurs (cpm/mg ash)
A	NaH_2PO_4	2	600 ± 13*	645 ± 68	6,134 ± 56	37.0 ± 2.8	398 ± 29
B	Na_2HPO_4	2	589 ± 22	572 ± 50	6,170 ± 82	32.8 ± 3.6	379 ± 31
C	Combination	2	459 ± 11	654 ± 67	6,335 ± 74	36.0 ± 3.0	428 ± 44
A	Na_2HPO_4	4	612 ± 11	705 ± 98	7,643 ± 53	45.6 ± 7.2	484 ± 38
B	Na_2HPO_4	4	572 ± 9	637 ± 60	6,691 ± 82	36.2 ± 2.5	445 ± 28
C	Combination	4	583 ± 9	632 ± 68	7,635 ± 55	42.6 ± 2.9	462 ± 14
A	NaH_2PO_4	6	561 ± 10	524 ± 69	9,442 ± 114	45.9 ± 6.0	611 ± 52
B	NaH_2PO_4	6	542 ± 14	461 ± 79	7,590 ± 77	40.8 ± 2.7	505 ± 43
C	Combination	6	541 ± 16	401 ± 114	7,555 ± 98	40.3 ± 4.8	489 ± 55
A	NaH_2PO_4	8	568 ± 10	432 ± 84	8,490 ± 44	54.4 ± 5.6	574 ± 26
B	Na_2HPO_4	8	504 ± 13	311 ± 101	7,567 ± 65	50.4 ± 2.0	443 ± 27
C	Combination	8	513 ± 10	428 ± 38	7,250 ± 79	46.8 ± 5.0	458 ± 27
A	NaH_2PO_4	24	551 ± 14	457 ± 44	3,744 ± 87	51.3 ± 2.5	522 ± 24
B	Na_2HPO_4	24	524 ± 7	226 ± 53	3,553 ± 123	48.1 ± 4.2	429 ± 28
C	Combination	24	501 ± 9	234 ± 53	3,816 ± 114	49.1 ± 8.0	428 ± 23
A	NaH_2PO_4	48	809 ± 14	275 ± 29	2,876 ± 45	69.4 ± 9.1	477 ± 40
B	Na_2HPO_4	48	542 ± 13	180 ± 41	2,444 ± 25	54.7 ± 6.2	431 ± 28
C	Combination	48	487 ± 9	193 ± 14	2,316 ± 57	54.2 ± 2.6	448 ± 34
A	NaH_2PO_4	72	899 ± 21	248 ± 26	2,880 ± 53	70.9 ± 4.8	492 ± 39
B	Na_2HPO_4	72	465 ± 8	214 ± 48	2,021 ± 43	54.2 ± 2.0	402 ± 24
C	Combination	72	579 ± 16	232 ± 59	2,162 ± 88	52.2 ± 3.0	440 ± 19
A	NaH_2PO_4	96	865 ± 22	224 ± 43	1,750 ± 47	66.7 ± 3.9	506 ± 38
B	Na_2HPO_4	96	506 ± 17	178 ± 54	1,367 ± 52	54.3 ± 2.7	330 ± 16
C	Combination	96	555 ± 16	212 ± 16	1,505 ± 49	55.8 ± 5.4	426 ± 34
D	Control	2-96	21.4 ± 4.5	29.8 ± 4.1	27.6 ± 4.0	4.1 ± 0.4	1.5 ± 0.2

* Standard error of the mean.

III. Long-term studies

1. Ref. 33 (Tripolyphosphate)

Rochester strain weanling rats were maintained on Fox Chow meal with 0.05, 0.5 or 5% sodium tripolyphosphate (TPP) supplementation for 2 years. Females on 5% TPP showed slight growth retardation during year 1 and conspicuous retardation during year 2. Males on 5% TPP were retarded for both years, with evidence of anemia in year 1. There was no difference between controls and the 0.05 and 0.5% levels. Relative splenomegaly was significantly elevated in males on 5% TPP, with modest increases for liver, testes, brain, stomach and heart; females were only randomly affected. Femur length and body weight for both sexes on 5% TPP were significantly lower than controls. Although the femurs were radiologically unremarkable and the Ca/P ash ratios normal, there was evidence of hydropexis and less organic material. Convoluted tubule dilation with interstitial and glomerular fibrosis and intertubular calcification ("chronic tubular nephropathy") was unique to the 5% group and was easily discriminable from the chronic pyelonephritis ("phosphate nephritis") of control, 0.05 or 0.5% diets. The carcinogenesis index was similar to frequencies expected for aging rats and did not vary among dietary groups.

TPP had no effect on fertility, litter size, growth survival, organ weights, splenomegaly or other gross and histological variables through F_2 at 0-0.5% levels.

2. Ref. 33 (Sodium trimetaphosphate)

Weanling rats were maintained on Fox Chow meal with 0.1, 1.0, or 10.0% sodium trimetaphosphate supplementation for 2 years. Males and females on 10.0% trimetaphosphate showed substantial growth retardation for both yrs., but males on 1.0% trimetaphosphate showed slight growth retardation up to the age of 6 mos., after which retardation became more pronounced; growth rate was normal during the second yr. No growth retardation occurred in females of this group. There was no difference between controls and the 0.1% level. Food consumption was normal at all levels. There was evidence of anemia in females of the 10.0% group, and a trace of urine proteins and reducing substances at all levels. Some organs

were heavier at the 10.0% level but the livers in both males and females were smaller than controls. Organ wts. were normal in the other groups. Femur length and body wt. for both sexes on 10.0% trimetaphosphate were significantly lower than controls. Although the ratio of femur length to body wt. was normal in the male, it was higher in the female. Levels of Ca and P of bones were elevated in both sexes in the 10% group, with less water and more ash in the male and the reverse in the female.

Kidney concretions, mostly in females, were present in the 0.1%, 1.0% groups, and controls, but not in the 10.0% groups. Trimetaphosphate had no affect on fertility, or litter size through F_2 at 10.0% levels. There was no correlation between the dietary level of trimetaphosphate and tumor incidence.

3. Ref. 33 (Sodium hexametaphosphate)

Weanling rats of both sexes were maintained on Fox Chow meal with 0.05%, 0.5% and 5.0% sodium hexametaphosphate (HMP) supplementation for 2 years. Males on 5.0% HMP showed a substantial growth retardation for both yrs., but the females were only slightly affected. Food consumption at 90 and 210 days was significantly greater in this group (both sexes) than in the others. HMP had no effect on blood; urine revealed only traces of protein and reducing substances at all levels of HMP supplementation. Kidney wt. was higher in both sexes in the 0.5% and 5.0% groups, but more so in the female of the latter group. Femurs appeared radiologically normal in all groups. Sixty-five percent of the rats in the 5.0% group exhibited an intertubular calcification ("chronic tubular nephropathy") as distinguished from the coexistent "chronic pyelonephritis" present in old rats. HMP had no affect on fertility or litter size through F_2 at 0.0-5.0% levels. There was no more correlation between the dietary level of HMP and tumor incidence.

The "no-effect" level of the four condensed phosphates when incorporated in the diet of rats over a period of 1 mo. was 2% in all cases. Extension of the feeding tests for 24 mos. indicated an absence of significant toxic effect at levels intermediate between

0.05 and 0.5% in the case of HMP, 0.5 and 5.0% in the case of TPP and 0.1 and 1.0% for trimetaphosphate.

IV. Special studies

1. Ref. 723 $(NH_4)_2HPO_4$ and $CaHPO_4$)

A 40 kg lactating ewe was fed a ration containing 3% $(NH_4)_2HPO_4$ for 45 days, beginning 25 days pre-parturition. Then a dose of 9 g of ^{32}P-labeled ammonium phosphate in aqueous solution was administered intra-ruminally. Total blood phosphorus (387 mg/l prior to the challenge dose) peaked at 467 after 47 hours and had returned to 398 at one week post-treatment. The ^{32}P-label, however, peaked at 21 hours and then rapidly dropped off. Total milk phosphorus varied only slightly and non-systemically during this one week period but ^{32}P again peaked at 21 hours, dropping off somewhat less abruptly than blood label.

Several weeks later the experiment was repeated on the same ewe, substituting $CaHPO_4$ (3.2%, to give an equivalent amount of P). Here total blood P was not systematically affected while the blood ^{32}P peaked between 21-32 hours and became unmeasureable by one week. Total milk P again varied non-systematically, but the ^{32}P peaked and remained essentially constant from hour 21 through the week.

The conclusion was that the salts were similarly absorbed and were utilized effectively for milk synthesis.

BIOCHEMICAL ASPECTS

I. **Break down**

No information available

II. Absorption-Distribution-Metabolism-Excretion

1. Ref. 43 (orthophosphates)

Rats of Long-Evans strain, 9-11 weeks old, were placed on a mainly corn-gluten diet containing 0.36% P and 0.34% Ca after a single p.o. administration of 20 - 50 mg P^{32}- labeled $CaHPO_4$, $Ca_3(PO_4)_2$ or $Ca(H_2PO_4)_2$ and p.o. or s.c. H_3PO_4 solution. Fecal and urine analyses were run at 24, 48 and 72 hours (Table I), tissue distribution assays at 72 hours (Table II) and rate studies at 12, 24 and 48 hours (Table III) with the amount of label excreted or accumulated calculated as percent of dose administered.

Table I. Urine and fecal recovery of P^{32} label after p.o. or s.c. administration of phosphoric acid and some calcium phosphates in rats, in percent of dose, at different times.

	H_3PO_4						$CaHPO_4$			$Ca_3(PO_4)_2$			$Ca(H_2PO_4)_2$		
	p.o.			s.c.			p.o.			p.o.			p.o.		
	hours						hours			hours			hours		
	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72
urine	5	7	8	2.5	5	5.5	8	12	14	10	12	14	10	15	15
feces	10	11	12	1	2	1.5	27	29	31	45	47	50	37	42	43

The excretory outcome of the p.o. acid would suggest both greater tissue absorption and retention than that for the phosphate salts, delivered in dry form by gavage. As indicated by table I, little of the label was excreted via either pathway after 48 hours. Approximately 3.5x more label underwent renal than gut elimination by 72 hours after s.c. treatment, which excretion rate was significantly below that for p.o. Table II shows tissue distributions for the various phosphates.

Table II. Tissue deposition of P^{32} label after p.o. or s.c. administration of phosphoric acid and some calcium phosphates p.o. in rats at 72 hours, in percent of dose per gram of fresh tissue.

	H_3PO_4		$CaHPO_4$	$Ca_3(PO_4)_2$
	p.o.	s.c.	p.o.	p.o.
liver	0.51	0.4	0.4	0.2
kidney	0.38	0.48	0.25	0.15
muscle	0.25	0.28	0.09	0.08
femur	3.1	3.5	1.3	1.3

The accumulation of p.o. acid was significantly greater than than the p.o. salts for kidney, muscle and, especially, femur. Serum label at 72 hours from acid or salts was below 0.1% of the dose and the amounts were roughly equivalent for each agent. Table III amplifies the phosphate salt data.

Table III. Tissue deposition and serum level of P^{32} label after p.o. administration of two calcium phosphates in rats at different times, in percent dose per gram of fresh tissue.

	Blood			Liver			Kidney			Muscle			Femur		
	hrs			hrs			hrs			hrs			hrs		
	12	24	48	12	24	48	12	24	48	12	24	48	12	24	48
$CaHPO_4$	0.18	0.17	0.06	1.4	1.1	0.3	0.9	0.8	0.3	0.2	0.2	.08	2.9	2.2	1.5
$Ca_3(PO_4)_2$	0.08	0.07	0.03	0.5	0.5	0.3	0.3	0.3	0.2	.07	.09	.06	1.3	1.6	0.7

Depot rates for each salt were maximized by 24 hours, after which at least a proportional decrease followed up to 48 or 72 hours.

2. Ref. 517 (H_3PO_4)

Eight subjects, four healthy and four diabetic, were given 60 drops of H_3PO_4 in 100 ml H_2O. Total blood phosphorus and blood pH of samples taken 1 hour after administration are not changed significantly from samples taken before administration, either with the healthy or the diabetic subjects.

3. Ref. 623 ($Ca_3(PO_4)_2$)

The solubilization of tricalcium phosphate by gastric juice is greater than that expected from only the acid content; i.e., $Ca_3(PO_4)_2 + 4H^+ = 3Ca^{+2} + 2H_2PO_4^-$. Some "calcium phosphate" reprecipitates in variable amounts after separation of the solid and liquid phases. The results are accounted for by postulating complexation of ionic

calcium with proteins or peptides present in gastric juice, thus shifting the above equilibrium to the right.

4. Ref. 626 (H_3PO_4)

Bile label was successively recovered from both ducti choledochus at 20 min intervals for 2 hrs and gall bladder evacuation at 2 hrs after injection of 1 ml $H_3P^{32}O_4$ in ear veins of 9 adult male rabbits. Serum and choledochus labels were maximized 10 and 15 min post-treatment, respectively, with subsequent exponential declines at equivalent rates. Gallbladder bile label was approximately intermediate between the duct maximum and the terminal duct level at 2 hrs. Terminal serum levels ranged between 25 and 430% higher than terminal bile values in all animals, such that serum/bile ratios varied from 5 to 21.5.

5. Ref. 106 (condensed phosphates)

Male rats, 24 - 28 days old, were maintained on a rachitogenic diet supplemented with sodium hexametaphosphate (HMP) or crystalline sodium trimetaphosphate. Calcium and phosphorus retention were 20 and 11% greater, respectively, on 7% HMP than trimetaphosphate, values compatible with relative complex-forming ability. A 3 1/2% supplement had little effect on Ca absorption. However, no x-ray detected rickets were found on any animals receiving P supplement.

6. Ref. 74 (Na_2HPO_4 and condensed phosphates)

Rats 4-6 weeks old were fasted for 16 hrs before receiving 1 ml $Na_2H^{32}PO_4$ (orthophosphate-oP), $Na_4{}^{32}P_2O_7$ (pyrophosphate-PP) or $(Na^{32}PO_3)_n$ monohydrate (hexametaphosphate-HMP) per 100 g body weight by gavage. Label recovery in gastrointestinal contents, gastric and gut mucosa and selected tissues is given in two tables on the following two pages.

The major portion of gastrointestinal label from both PP and HMP shifted site at about 1 hour post-treatment: PP showing an even distribution through each gastrointestinal segment and HMP showing a dramatic change from stomach to colon accumulation within 30 minutes. By 2 hrs the colon levels from oP and PP were equivalent, indicating a higher oP than PP turnover rate between 1-2 hours. Colonic harvest predominated for each phosphate from 2-6 hours (75 to 95%), but only the label from HMP continued to rise (32.5 to 44.5%). Since oP and PP recoveries were metastable during that period, with evidence of tissue transit for both, gut resorption (and

Percent label recovery from rat gastrointestinal contents (GIC), gastric and intestinal mucosa and other tissue at different times, from oral orthophosphate (oP) or pyrophosphate (PP) administration

Time (hrs)	1/4		1/2		1		2		4		6	
GIC	op	PP	op	PP	op	PP	op	PP	op	PP	op	PP
Stomach	35	23	19.5	18.5	21	6.7	1.3	0.5	0.1	0.1	0.1	0.1
Intestine-U*	4	2	2	5	1	5.5	0.8	0.4	0.3	0.3	0.05	0.3
Intestine-M*	5.5	2.5	4	8	3.7	5	3	1.5	0.3	0.5	0.05	1.1
Intestine-L*	2	4	4	6	6	8	3.5	6.3	1.8	1.9	0.4	0.3
Colon	0.9	1.3	0.4	1	8	11.7	22	22.7	21.5	23.5	26.5	24.5
GIC total (approx.)	47.5	33	30	38.5	39.5	37	30.5	31.5	24	26	27	26
Mucosa												
Stomach	8	8.3	11.5	8	8	4.5	1.2	1.1	0.8	0.9	0.6	0.6
Intestine-U*	5.3	6.7	5	7.5	4	9.5	3	3.2	2.5	2.5	1.7	2
Intestine-M*	6	6.7	5.8	7.5	5.7	6.3	2.8	2.5	1.8	1.7	1.6	1.5
Intestine-L*	4.7	11	7.5	9.5	11	8	6.3	6.4	1.3	1.4	1.2	1.4
Colon	1.8	1.2	1.6	1.3	5	3	4.2	5.7	6	4.3	5.5	6.5
Mucosa total (approx.)	26	34	31.5	34	33.5	31	17.5	19	12.5	11	10.5	12
Liver	0.9	1.5	3.5	2.2	3.8	3.5	4.3	3.6	3.9	4	3.6	3.6
Kidney	1.3	0.6	0.4	0.7	0.8	1.5	1.2	0.9	0.9	0.8	0.7	0.8
Tibia	0.2	0.1	0.2	0.4	0.5	0.7	0.8	0.8	1.2	1.1	1.3	1
Muscle	0.04	0.04	0.02	0.1	0.12	0.05	0.09	0.05	0.07	0.04	0.06	0.1
Blood (whole)	0.2	0.4	0.2	0.3	0.35	0.35	0.4	0.3	0.4	0.3	0.26	0.28

*"U", "M" and "L" refer, resp., to roughly equivalent upper, middle and lower segments of the small intestine

Percent label recovery from rat gastrointestinal contents (GIC), gastric and intestinal mucosa and other tissue at different times, from oral hexametaphosphate administration

Time (hrs)	1/4	1/2	1	2	4	6
GIC						
Stomach	17	27	7.3	2	0.3	0.1
Intestine-U*	2	0.8	0.5	0.6	0.2	0
Intestine-M*	7	2	1.7	1.3	0.5	0.1
Intestine-L*	2	11	3.5	3	4	3
Colon	1.2	0.3	22.5	32.5	33.5	44.5
GIC total (approx.)	29.5	41	35.5	39.5	38	48
Mucosa						
Stomach	6	8	4.2	2.2	0.4	0.3
Intestine-U*	3.4	4	2.5	2	0.8	0.5
Intestine-M*	13.5	6	5.7	2.6	0.5	0.4
Intestine-L*	14.5	17	7.8	5.8	2	0.7
Colon	0.5	0.6	13	9.7	11.5	6.8
Mucosa total approx.	38	35.5	33	22	15	9
Liver	0.8	0.4	0.5	0.3	0.7	0.7
Kidney	0.6	0.07	0.2	0.3	0.3	0.2
Tibia	0.02	0.05	0.07	0.2	0.2	0.2
Muscle	0.005	0.02	0.04	0.03	0.05	0.02
Blood (whole)	0.03	0.04	0.04	0.04	0.05	0.05

*"U", "M" and "L" refer, resp., to roughly equivalent upper, middle and lower segments of the small intestine

utilization) from oP and PP was assumed to be roughly equivalent. Significantly lower serum label from HMP and higher initial mucosal capture than oP or PP would tend to confirm that significant increments of label from HMP is refluxed back into the small gut lumen without absorption, for fecal elimination.

7. Ref. 324 (polymeric phosphates)

Fasting adult rabbits were injected i.v. with either cyclic sodium trimetaphosphate or cyclic sodium tetrametaphosphate (25 mg P/kg in both cases). Both compounds produced only a slight increase in the serum concentration of orthophosphate, which occurred late in the experiment. The unhydrolyzed compounds appeared promptly in the urine (exponential removal from blood) and excretion was essentially complete after four hours. The increase in orthophosphate in the urine above control amounted to not more than 2% of the unhydrolyzed compounds appearing there.

When male rats were injected i.p. (50 - 110 mg P/kg) with the same two compounds, 90% of the cyclic metaphosphate appeared unhydrolyzed in the urine after 24 hours, with no increase in excretion of orthophosphate detected. When the Ca salts were administered the amount dropped to 48 - 77% of unhydrolyzed compounds in the urine, with some increase in urinary orthophosphate noted.

Unlike the two cyclic metaphosphates, when the sodium or calcium salts of acyclic hexametaphosphate (all cyclic contaminants removed) or tripolyphosphate were administered to rabbits, significant hydrolysis was noted. Serum orthophosphate concentration showed a rapid and sustained rise after i.v. injection of calcium sodium hexametaphosphate, with a rapid decrease in polymeric material. Injection i.v. of either hexametaphosphate or tripolyphosphate yielded twice as much orthophosphate in the urine as unhydrolyzed (or partially hydrolyzed) phosphorus.

In rats, also, parenteral doses of these two linear oligomers promoted a large excretion of orthophosphate with correspondingly less polymeric phosphorus present in the urine.. The total excretion of phosphorus from s.c. doses of calcium hexametaphosphate was always low, because, it remained as an unmobilized deposit for long periods of time.

When either the cyclic or the linear phosphates were administered orally, however, significant amounts of orthophosphate and almost no polymeric phosphates appeared in the urine of rats or rabbits.

III. Effects on enzymes and other biochemical parameters

1. Ref. 320 (sodium hexametaphosphate)

Various amounts of sodium hexametaphosphate (HMP) were incubated at pH 7.4 with sensitized ox rbc after HMP treatment with heterologous serum. Only partial hemolysis occurred at 0.89% HMP, while higher doses were negative. In conjunction with independent evidence, the complement suppression by HMP was due to a combination of HMP and serum protein and not the removal of calcium ions from serum. Dibasic sodium phosphate was without serum effect, while pyrophosphate elicited slight complement loss by increased protein dispersion. It is suggested that the complex HMP anion competes with protein anions for basic groups on serum proteins, forming insoluble precipitates.

2. Ref. 527 (sodium hexametaphosphate)

The *in vitro* clotting of human blood was inhibited completely by 0.1% sodium hexametaphosphate; lower concentrations only slowed clotting. This effect is probably a result of the chelation of calcium ion by hexametaphosphate.

3. Ref. 44 (sodium metaphosphate)

Lysine rich histone (f_1) from calf thymus, at 5 μg/ml, stabilizes boar sperm cells, as measured by their mobility after 48 hours storage at 18-22°. Addition of 125 μg/ml sodium metaphosphate, simultaneously with histone f_1 or after 15 - 30 min., significantly reduces the activity of the sperm cells. However, addition of sodium metaphosphate after 45 min. and later has no influence on the stabilizing action of histone.

4. Ref. 325 (sodium polyphosphate)

The effect of sodium polyphosphate preservative on the growth of *Bacillus* sp. from endospores was followed by phase contrast microscopy. Yeast glucose agar containing sodium polyphosphate (mixture of molecules (containing 2 - 15 or more P atoms) was solidified as a thin film on microscope slides and the slides were then inoculated with drops of a suspension containing 10^7 endospores per ml sterile distilled H_2O. After incubation in a warm chamber the slides were examined periodically. In 0.2% polyphosphate the emerging vegetative cells appeared distorted and failed to divide. In 1% inhibitor, growth ceased before rupture or lysis of the spore wall and in higher concentrations germination of the endospores was reduced. Sodium polyphosphate was 1.5 to 2 times

more effective at pH 6 than pH 7.

5. Ref. 827 (sodium tripolyphosphate)

The growth of P-1 phage in *Microbacterium ammoniaphilum* is inhibited by addition of sodium tripolyphosphate (NaTPP). NaTPP (1 mg/ml throughout) has no phagicidal effect on free phage and has no effect on synthesis of RNA, DNA or protein in infected cells. Addition to the culture prevents infection, only if administered within the first 5 min. of addition of phage. This first 5 min. is the period in which absorption to the cell and injection take place. If NaTPP is added to the bacteria simultaneously with ^{32}P labeled phage, 72% of the ^{32}P can be removed by a blendor treatment after culturing the mixture for 30 min at 30°. If no NaTPP is added with ^{32}P labeled phage, only 10% of ^{32}P can be removed by this treatment. The effect of NaTPP is to prevent injection into the bacterial cell once the phage is absorbed, and leave it locked on the cell surface, where it can easily be removed.

6. Ref. 644 (sodium pyrophosphate)

Sodium pyrophosphate (PP) solution (pH 7) at 0.002M induced 28% chromosome breakage, gaps or chromatid breaks in secondary root cells of *Vicia faba* under anaerobic conditions (1% oxygen). Higher molarities (0.004 and 0.006) were lethal at 1% oxygen, while there were 22% gaps at 0.005M and 3% O_2, and 15% gaps at 0.005M and 20% O_2. Anaphase suppression, cytoplasmic shearing, reduction in high activity of S-interphase, and a plateau in the response of strand recombination rate to PP dose above 0.05M, would also implicate PP in both RNA and DNA synthesis abnormality. Anaerobiosis as a sufficient condition for cytogenetic deficit would suggest reduced deoxyribonucleoside triphosphate pools, which effect on lower nucleic acid polymerization is potentiated by PP.

7. Ref. 750 (condensed phosphates)

Various phosphates were injected s.c. or i.p. in non-toxic 0.5 ml doses (mg P?) into Swiss mice with assorted transplanted tumors, whose arrest was assayed in percent by weight. Potassium polymetaphosphate inhibited carcinoma 755 and leukemia 1210 by 50% and sarcoma 180 by 30%. Tetrasodium pyrophosphate, sodium hexametaphosphate and tripolyphosphate and potassium metaphosphate were 30% inhibitory of Krebs 2 tumor. Inhibition of tumor intracellular DNase could explain tumor arrest.

8. Ref. 673 (polyphosphate)

Endogenous respiration of Ehrlich Ascites tumor cells in suspension, in the presence of various concentrations of polyphosphate, was measured by the method of Warburg. Polyphosphate inhibition of respiration increased with concentration. At 50 min. the amount of O_2 taken up was 5x less than without 3.3 mg/ml of polyphosphate. The inhibition is counteracted by M/10, Mg^{++} or Fe^{++} and partially by other ions, but no clear relation between chelation by polyphosphate and inhibition could be observed.

9. Ref. 966 (pyrophosphate and tripolyphosphate)

The bactericidal effect of 50 ppm of alkyl dimethyl ethyl benzylammonium chloride on *Pseudomonas fluorescens* at pH 9.2 was potentiated ca. 2×10^4 times by $Na_4P_2O_7$ (NaPP) or sodium tripolyphosphate at the concentrations used in detergent sanitizers, after 30 sec. contact; and the virucidal effect of 25 ppm of the quaternary ammonium salt against filtrates containing phage 144-F of *Streptococcus cremoris* after 30 sec. contact was potentiated 10^3 times by 200 ppm NaPP at pH 8.0, as measured by the plaque technique. Lower concentrations of NaPP were less virucidal.

10. Ref. 479 (sodium hexametaphosphate)

"Taka-phosphatase" converts sodium hexametaphosphate into orthophosphate. Phosphatase from canine liver or kidney or from yeast also exhibits somewhat weaker activity on hexametaphosphate.

11. Ref. 652 (orthophosphate, pyrophosphate)

Alkaline phosphatase from the intestine of a dog was subjected to prolonged (20 days) dialysis against double-distilled H_2O until the enzyme was completely inactivated. Addition of divalent ions was effective in restoring activity, well above predialysis levels (Fe - max 420% of predialysis level, Zn - 420%, Mn - 478%, Ca - 490%, Mg - 640%) but, only, if the enzyme was first incubated for two hours with d,l-alanine at 37° and pH 8.8.

Orthophosphate, pyrophosphate and fluoride all strongly inhibited reactivation of enzyme activity by alanine incubation and divalent metal

ion addition, but, only, if these inhibitors were incubated with the inactivated enzyme for two hours at 37° and pH 8.8, prior to alanine treatment, suggesting that orthophosphate, pyrophosphate and alanine compete for the same enzyme site.

12. Ref. 91 (pyrophosphate)

Mg^{+2} catalyzes the hydrolysis of pyrophosphate by rat erythrocyte pyrophosphatase. There is a sharp rise in the hydrolysis rate as the $Mg^{+2}/P_2O_7^{-4}$ ratio increases from 0.4 to 1.6 and eventually reaches a maximum at 3.81. A rise in pyrophosphate concentration accelerates the rate of enzymic hydrolysis as long as Mg^{+2} is in excess. The rate is slowed by addition of pyrophosphate when $P_2O_7^{-4}$ is in excess, because, Mg^{+2} is witheld from active enzyme centers by the formation of a $MgP_2O_7^{-2}$ complex.

13. Ref. 707 (sodium phosphate)

Immediately after the start of the experiment or ^{60}Co radiation 125 gm male Sprague-Dawley rats received sodium phosphate (2.4 mg P/kg) with 5μmole/kg tris at pH 7.2 i.p. They also received 1 mg/kg of colchicine at this time and again 2.5 hours later to stop cell mitosis at metaphase.

Phosphate treatment significantly accelerated the rate of mitosis in the thymus and bone marrow of irradiated and unirradiated animals for at least 6 hours. The total Ca level of the blood was depressed after phosphate injection and very slowly recovered, but, the level of ionized Ca, after a short-lived initial depression, climbed well above normal limits, in compensation. The high level of ionized Ca in the blood is believed to be the cause of the increased rate of mitosis.

14. Ref. 991 (sodium phosphate)

Normal and immune canine sera possess a substantial protease inhibiting activity, which caused protein levels to rise ca. 1 - 2% in solutions of serum incubated for 48 hours at 37° with *Bacterium vulgaris*. When 2.5 mg/ml sodium phosphate was added to the incubation mixture bacterial protease activity was increased and the serum protein level fell by ca. 4%.

15. Ref. 132 (orthophosphate)

A medium simulating the ionic composition of the intracellular fluid; i.e. high in K^+ (130 mmole/l) and Mg^{++} (20 mmole/l), was found to promote glycogenesis by rat liver slices from a 1% glucose solution. The addition of phosphate to the medium (Mg/P of 2/1 to 1/4) did not inhibit glycogen synthesis. However, when phosphate (10 mmole/l) and succinate (87 mmole/liter) were combined with the medium no net synthesis occurred.

16. Ref. 220 (H_3PO_4)

The erythrocyte count of dogs was lowered 31 - 43% by removal of 30 cc/kg of blood from the jugular vein, and the regenerative effect of administration of 2 ml concentrated H_3PO_4, daily and perorally, was studied.

Phosphoric acid treatment caused the erythrocyte count to return to normal sooner (17 days) than in untreated controls (27 days). A greater number of heterochromatic erythrocytes and nucleus-containing erythrocytes (43%, compared to 20%), and, a more pronounced "post hemorrhagic" leukocytosis (especially myelocytes and granulocytes) were observed after this treatment. The effects were also observed with H_2SO_4 and HCl.

17. Ref. 831 ($CaHPO_4$)

Evidence indicated that $CaHPO_4$ is the important phosphate of calcium in serum. The solubility product of $CaHPO_4$ was determined at the ionic strength of serum. Various concentrations of calcium ion and phosphate were shaken with excess crystalline $CaHPO_4$ until equilibrium was reached. K's.p. $CaHPO_4$ = 3.2×10^{-6} at room temperature and 3.4×10^{-6} at 38°.

18. Ref. 760 (orthophosphate)

Embryonic bones and teeth, before onset of ossification, contain an excess of phosphorus over calcium. Treatment of embryonic skeletal parts of sheep (marrow removed) with 2N HCl reveals a Ca/P much lower than expected for $Ca_3(PO_4)_2$. Treatment with trichloroacetate solution reveals only traces of phosphate esters and dissolution in potassium glycerate solution indicates that phosphate exists in a very readily ionizable form, probably fixed to protein.

19. Ref. 48 (orthophosphate)

Samples of fresh and processed fruit juices, various milks and phosphate-rich animal or vegetable products were incubated at body temperature for 1 hr in 1:1 ratio with simulated gastric juice solutions. (2% pepsin in 0.4% HCl, pH 1.7). Excepting only a certain few mushroom preparations, wines and commercial mashes, free orthophosphate increased from 5 to 300% after digestion by the model system.

20. Ref. 792 (sodium metaphosphate)

Mature male rats received an adequate diet containing 8% sodium metaphosphate for 7 months or until exitus. Radiological and biochemical assay during life showed gradual bone decalcification with focal osteoclasis, renal calcium deposition (mainly medullary), inorganic phosphaturia and significant parathyroid hypertrophy and hyperplasia. Histological evidence of metastatic calcium deposits was extensive in various tissues, including renal tubules and long-bone periosteum and endosteum.

21. Ref. 696 (sodium orthophosphate)

Hypercalcuria and renal stones were observed in ten patients, three with hyperthyroidism, one each with sarcoidosis and Paget's disease and five with idiopathic hypercalcuria. Oral treatment of all patients with ca. 90 mg/kg/day of sodium phosphate (pH 7) for 18 days caused the daily urinary Ca level to fall by an average of 126 mg, with increased urinary phosphate.

22. Ref. 758 (calcium orthophosphate)

The inhibitory effect of various urine protein fractions, from normal and stone-forming men, upon homogeneous nucleation of calcium phosphate crystals was investigated. The largest mean difference between normal and stone-forming men in any single protein fraction amounted to no more than 5%.

23. Ref. 122 (orthophosphate)

A fully grown male rabbit was fasted for 18 hours and then received s.c. phosphate (141 mg P/kg) in a minimum of H_2O. At the end of one hour serum phosphate had risen 140%. At the end of two hours samples were unobtained due to the collapsed state of the animal.

24. Ref. 203 (H_3PO_4)

Food rations containing cotton seed hulls and phosphoric acid fed to one of eight lots of steer were more effective in preventing the formation of renal calculi than any of the other rations containing sumac silage with ammonium chloride, hyaluronidase, or phosphoric acid. Three of these lots contained cottonseed hulls that replaced a small portion of the silage. Cottonseed hulls in rations containing 52 ml phosphoric acid proved 86% more effective than its equivalent ration (silage-phosphoric acid), 99% more than the other three lots containing seed hulls, 97% more than those containing the sumac silage, 94% and 97% more than the "silage" and "hull" control rations, respectively.

25. Ref. 79 (orthophosphate)

The effect of sodium phosphate treatment on renal calculi and idiopathic hypercalcuria of 10 patients was studied. All were hypercalcuric and 9 of 10 were passing stones. An oral dose of 3.3 - 6.6 gm sodium phosphate was given daily for 4 - 5 months. By this treatment urinary Ca was lowered an average of 51% (brought to within normal limits in 6 cases), and all 9 with renal stones showed a marked reduction in the number passed. Renal phosphate clearance fell in some but rose in others. The only harmful effect noted was a mild diarrhoea, which was generally abolished by altering the dosage. There was no radiological manifestation of extraskeletal calcification or alteration in the function in any other system.

26. Ref. 662 ($Ca_3(PO_4)_2$, Na_3PO_4)

Equimolar supplements of $Ca_3(PO_4)_2$ and Na_3PO_4 were given every 10 min for several hours in 10 g forcemeat diets to Heidenhain pouch dogs. The curves of gastric juice stimulation are suggested by the following table:

Secretion (ml) induced by	Time (hr)											
	0.5	0.66	0.75	1.0	1.25	1.5	1.75	2.0	2.25	2.5	2.75	3.0
$Ca_3(PO_4)_2$	1.5	2	1.9	1.1	2.5	2.8	2.0	0.9	2.5	2.5	1.5	2.5
Na_3PO_4	1.2	1.1	2.1	0.9	2.0	1.2	1.7	1.5	1.6	1.6	1.8	1.5

Independent dietary trials with other calcium salts confirmed that phosphate, not calcium, was the secretagogue. The relative ineffectiveness of Na_3PO_4 was attributed to unknown factors responsible for different degrees of charge-dependent ion penetration through gastric mucosa. Increasing phosphate dietary concentrations elicited graded dose-responses in secretory rate, which even remained at plateau for 1 hr post-treatment and suggested the presence of a phosphate concentration gradient between gastric contents and gastric mucosa.

27. Ref. 873 (Na_2HPO_4)

The average gastric emptying time after ca. 54 and 108 mg/kg dibasic sodium phosphate had been fed four dogs (8.6 - 9.8 kg) was 5.64 and 5.51 hrs. respectively. These times were insignificantly elevations over the control value of 5.25 hrs.

28. Ref. 720 (sodium orthophosphate)

Rabbits, subjected to daily i.p. injections of increasing concentration of sodium phosphate (at pH 7.2) for 3 weeks to 2 months, exhibited hypertrophy of the parathyroid. The weight of the organ increased from 2.83 mg/kg for controls to 3.94 mg/kg for those administered sodium phosphate. Dogs subjected to the same hyperphosphatemia via i.v. administration only, showed only slight hypertrophy. Dose levels were not given.

29. Ref. 885 [$Ca(H_2PO_4)$]

Patients were suffering from spatic constipation and intestinal swelling, diarrhea, stomach aches and other symptoms traceable to inhibition of intestinal coliform bacterial growth. Daily oral administration of an aqueous solution of 1 gm $Ca(H_2PO_4)_2$ for 3 - 4 weeks promoted remission in 60% of the cases.

30. Ref. 323 (sodium hexametaphosphate)

Manometric assay of CO_2 arterial plasma content and urine ammonia levels and titratable acidity confirmed the presence of an acute acidosis following hydrolysis of i.p. or i.v. sodium hexametaphosphate (29 mg P/kg) in rats. Acid excretion was 41% higher than controls during the first 24 hours, with the rate of acid formation sufficiently rapid to elicit a moderately acute systemic acidosis within the first hour and hyperpnea within a few minutes. The control level of ca. 29 meq/l arterial CO_2

was markedly reduced by 192 mg P/kg sodium hexametaphosphate i.p. (the LD_{50}) to a median of ca. 18 meq/1. The effect of calcium hexametaphosphate s.c. containing the same amount of phosphorus was much less marked (median ca. 26 meq/1).

Hypocalcemia was measured relative to extent of Q-T interval prolongation in the EKG. A pentobarbitalized rat received the sodium salt i.v. for one minute. Results are shown in the following table:

Sodium hexametaphosphate induced Q-T interval prolongation

Time (min.)	Description	Heart rate	Q-T (100) / R-R
0	normal	327	42%
1/2	incipient hypocalcemia	325	51
1 1/4	maximum hypocalcemia	310	59

Average changes in Q-T (relative to cycle length, RR) in pentobarbitalized rats receiving other phosphate polymers by femoral vein infusion at a constant rate of 6.2 mg P/min for 10 mins. were as follows: the [(Q-T)(100)/R-R] rose from 42 to 55 in 1/2 min for sodium tripolyphosphate, from 42 to 62 in 1 min after sodium hexametaphosphate, from 38 to 66 in 1 1/2 min after sodium pyrophosphate (NaPP), from 35 to 39 in 7 min after sodium orthophosphate and from 39 to 40 in 6 min with sodium tetrametaphosphate, with a decrease from 40 to 39 in 8 min for sodium trimetaphosphate. Onset of bradycardia, A-V block or arrhythmia leading to cardiac arrest, was simultaneous with a Q-T interval representing 60% of the cardiac cycle. Calcium-sodium hexametaphosphate (prepared from sodium hexametaphosphate and containing sufficient $CaCl_2$ to yield a Ca/P mol ratio of 0.3) elevated the Q-T(100)/R-R quotient from 38 to 57 within 10 minutes. The LD of the Ca-Na salt was 3-7x higher than the Na salt alone, whose LD was 48 mg P/kg. Moreover, the combined salt elicited accelerated respiration, cyanosis, pulmonary edema and a myocardial activity outlasting respiration. In contrast, respiration decelerated and the apical cardiac pulse was arrested before or simultaneously with respiration arrest during sodium HMP infusion.

The mechanism of mortality evidently differed in the Na and Na-Ca cases, although plasma calcium was depleted by both salts.

Single i.p. Na HMP doses as high as 250 mg P/kg, elicited no EKG evidence of hypocalcemia. A dose (LD_{50}) of tripolyphosphate (135 mg P/kg) elevated the [Q-T(100)/R-R] only from 35 to 42 after 3 hours. Significant prolongation of Q-T was registered by survivors after 50 mg P/kg i.p. NaPP: [Q-T(100)/R-R] rose from ca. 33 to 46 at 1 hour and lasted for 1 hour before falling to 44 at 3 hours with subsequent normalization. The Q-T/R-R quotient rose from ca. 36.5 to 56 among rats receiving 50 mg P/kg NaPP i.p. but with fatal outcomes; in these cases, Q-T prolongation was irreversible and signalled bradycardia and arrhythmia. The quotient for 150 mg P/kg NaPP i.p. rose from 40 to 56 within one hour.

31. Ref. 764 (sodium orthophosphate)

The effect of sodium phosphate upon calcemia in normal and spasmophilic infants was determined. When increasing doses of anhydrous sodium phosphate were administered orally to two spasmophilic infants a corresponding fall in blood Ca level was observed. At high enough dose level spasms resulted. When much higher dose levels (5 x 2 gms in 2 days) were administered to normal infants the drop in Ca level was negligible and no spasms were observed. When $CaCl_2$ is administered to normal infants no changes in blood Ca are observed, but, there is a significant rise when administered to spasmophilic infants. It is concluded, therefore, that the spasmophilic infants lack the mechanism for Ca regulation.

32. Ref. 225 ($Na_4P_2O_7$ and NaH_2PO_4)

Fifteen cats, artifically respired with ambient air to the extent that spontaneous respiratory movements were barely abolished, received energy rich phosphates via carotid catheterization and both ipsi- and contralateral carotid nerve discharges were assayed. Sodium pyrophosphate (58 μg/ml) failed to elicit an early (latent period 2 sec. or less) chemoceptor firing when all pressoreceptors were severed. Intense chemoception followed only after 3 minutes with a "late" progressing - regressing of ca. 15 sec. duration.

In animals with pressoreceptors intact 100 μg/ml failed to alter the tonically discharging fibers for 7 sec.; normalization followed 10 sec. post-treatment with subsequent normal chemoceptor discharge and elevated rhythmicity of tonically activated pressoreceptors. NaH_2PO_4 evoked mild "late" excitation only with much higher doses (up to 2.4 mg/ml). Both phosphate compounds elicited continuous (not pulse-synchronous) repetitive activity of medium voltage pressoreceptor spikes several seconds post-injection. It was concluded that degree of chemoceptor stimulation could be correlated with amount of high energy phosphate bond available.

33. Ref. 133 (orthophosphate and condensed phosphates)

The action of several phosphorus containing compounds on the tibialis anterior muscle (6-7 gm range) of 2.8 - 3.5 kg decerebrated cats, was examined. Injections of the subject compounds in 0.5 ml physiological saline, followed immediately by 0.5 ml Tyrode solution, were made into the distal part of the anterior tibial artery, the proximal part being closed by traction on a ligature when the substances were applied. Muscle tension and the "action potentials" were recorded simultaneously as measures of muscular activity.

Close arterial injection of ATP, sodium tripolyphosphate and sodium pyrophosphate in the range of 0.1 to 1.25 x 10^{-6} mole/gm of muscle evoked strong, prolonged tetanic contraction of the muscle, which increased in tension and duration with increasing dosage. The first injection of sodium pyrophosphate, however, had a damaging effect on the muscle and the muscle was less able to elicit a response to a second dose. ATP and sodium tripolyphosphate did not produce this effect.

Injection of 0.58 to 8.1 x 10^{-6} mole/gm muscle of adenylic acid produced brief tetanic contractions. When administered with sodium pyrophosphate (0.58 + 0.58 x 10^{-6} mole/gm muscle) prolonged response of the ATP type appeared; 1.7 to 7.0 x 10^{-6} mole/gm of ADP also produced a short tetanus-like response which was potentiated by orthophosphate (1.0 + 1.0 x 10^{-6} mole/gm), although orthophosphate alone evoked no contraction at a concentration of 1.7 x 10^{-6} mole/gm muscle.

The active substances evoked contraction, also, after complete curarisation of the muscle.

34. Ref. 591 (NaH_2PO_4)

White rats weighing 130 - 170 g received an aqueous solution of NaH_2PO_4 (30 mg P/kg) i.p., were sacrificed at intervals up to 24 hours and the gastrocnemius was analyzed. Phosphocreatine was 58% higher than control levels within 2 hours, falling to 36% at 6 hours and normalization at 24 hours. After 2 hours glycogen content showed an 18% rise, with subsequent fluctuation around control levels. In the initial 60 minute period the lactic acid level rose 61% and returned to normal or slightly below by 24 hours. Sciaticotomy failed to alter the maximal 2 hour phosphocreatine, implying a direct phosphate effect on muscle creatine-phosphocreatine, with subsequent phosphocreatine accumulation.

35. Ref. 340 (H_3PO_4 and Na_3PO_4)

The effect of several phosphorus containing compounds on the gastrocnemius-soleus muscle of cats was measured 8 - 60 days after denervation of this muscle by section of the sciatic nerve. Close arterial injection of 1 mg/kg or less in physiological saline of H_3PO_4, Na_3PO_4, Sarin, isopropyl methyl phosphonofluoridate (DFF), Na_2PO_3F, $Na(iPr)PO_3F$(iPr = isopropyl) and $Na(iPr)_2PO_4$ elicited muscle contraction in most animals after one injection. When the cholinesterase concentration in motor endplates was analyzed by Koelle staining in the presence of H_3PO_4 and Na_3PO_4 neither were found to possess anticholinesterase activity.

36. Ref. 102 (orthophosphate)

Phosphate, being a decalcifying agent, provoked autorhythmic reflex of tonic muscle of the frog. Stimulation of the central end of the homolateral sciatic nerve by two AC discharges, 5 - 10 m sec. apart, caused reflex of semi-tendonous tonic muscle of the thigh. The "after discharge" of the tonic muscle caused by this stimulation, before and after injection of 0.25 cc of 0.5 M phosphate at pH 7.4 into the aorta, was taken as the measure of autorhythmic response of this muscle. Response to phosphate was due to action on the spine since all direct action on the muscular area itself was prevented by ligating the upper end of the iliac artery before injection. The increase in autorhythmic response caused by phosphate began two min. after injection, was several times normal after 100 min and was still detectable 24 hrs after injection. No response was observed at this concentration by the "nontonic" (author's term) triceps of the thigh.

37. Ref. 819 (NaH_2PO_4)

Female Sprague-Dawley rats (100 gm) were subjected to various stressors, either before or after s.c. injection of 1 mM NaH_2PO_4 in 2 ml H_2O by the "pneumoderma technique". The effect on muscular contractions was examined. When groups were subjected to forced exercise 1 day before and just before NaH_2PO_4 treatment, forced exercise just after treatment, forced restraint in the prone position for 17 hours prior to NaH_2PO_4 treatment, or 30 min periods of 115V AC shock every 20 sec. just after and two hours after treatment, a sharp "flick" of the finger on the sacral region induced a severe tetany-like condition with constant tremor and attacks of generalized, clonic convulsions, which caused death in 40, 10, 20 and 70% of the animals in each group, respectively. Bilateral vagotomy, induced hemorrhage, iliac ligation or s.c. injection of 6 mg in 0.2 ml H_2O of pentamethylenetetrazol and subsequent NaH_2PO_4 treatment or NaH_2PO_4 treatment and subsequent trauma from pinching a thigh muscle were found to cause little or no abnormal contraction. However vagotomy with NaH_2PO_4 treatment caused 70% mortality. NaH_2PO_4 treatment alone had no abnormal effect.

38. Ref. 317 (Na_2HPO_4)

The infusion of 1 liter of 1% Na_2HPO_4 (pH 7.4) into one normal subject in the supine position increased urinary excretion of Na(> 100%) and K(> 150%) and decreased excretion of chloride. Serum protein and chloride decreased slightly, and phosphate showed a large increase (ca. 900%).

When infused while the subject was standing quietly, urine Na concentration increased but K concentration was unaffected.

39. Ref. 578 (H_3PO_4)

Method

Species: man

sex: healthy males

age: 20 - 56

route: p.o.

vehicle: diet (in jam)

Group A (4 men) received 2.37 g daily of 84% H_3PO_4 for one week (200 mg/kg). Group B (2 men) received 3.16 g daily for four weeks (1060 mg/kg total dose). Group C (4 men) received 0.8 g daily for eight weeks (538 mg/kg total dose). All groups were kept on a standard, balanced diet, low in phytic and oxalic acids, averaging 110 g protein/day.

Results

No ill effects were observed; the highest dose level (Group B) "was felt as a strain".

In groups A and B a slight, almost statistically insignificant hypocalciuria was noted, with return to normal after cessation of phosphate loading. Group C showed no change.

In group A urinary pH dropped 0.2 - 0.3 units, preformed ammonia increased slightly (by 50 - 100 mg daily). No acidemia was indicated since CO_2-combining capacity of plasma was unaffected, as were urinary excretion of potassium and total fixed bases. In B, urinary pH dropped 0.6 - 0.7 units and NH_3 increased 100 - 300 mg daily.

With groups A and C detailed hematology was investigated, with no significant changes found in Ca, inorg. P, alk. phosphatase, Hb, NPN, CO_2 - combining capacity or total serum proteins. Urinary excretion of organic acids and N were also unaffected. Group B was not studied for these parameters.

Actual calcium balances were unaffected in all groups.

40. Ref. 306 ($Ca_3(PO_4)_2$)

Atheromas are known to calcify, so the degree of adsorption of cholesterol (0.16 mg in 1 cc) onto 20 mg $Ca_3(PO_4)_2$ was examined in the presence of 20 mM triethanolamine - nonanoate. 85% was adsorbed in 30 min. Adsorption was less with other concentrations and agents.

41. Ref. 718 ($CaHPO_4$)

The increase in salivary phosphate concentration on chewing a $CaHPO_4$-rich gum, which rose 6x within 15 min above pre-mastication levels, was associated with the $CaHPO_4$-containing salivary suspension itself and not with the anion in solution.

IV. Drug interactions

1. Ref. 241 (NaH_2PO_4)

Many substances were found to be effective in increasing serum levels of Aureomycin in rats. Female rats (approximately 125 gm) from the Lederle colony were administered, simultaneously, 25 mg Aureomycin·HCl and 1.6 gm/kg NaH_2PO_4 in aqueous solution by gavage. The serum level of Aureomycin after 4 hours was 10.4 μg/ml. This was 5.2 times the level of controls, which received no adjuvant NaH_2PO_4. No attempt was made to vary phosphate with Aureomycin dosages.

2. Ref. 821 (NaH_2PO_4)

Sprague-Dawley rats received s.c. 50 μg methyl-9α-chlorocortisol as the acetate in 0.2 ml H_2O, once daily, and 2 ml of a 15% aqueous solution of NaH_2PO_4 by gavage, twice daily. Nephrocalcinosis was observed in all rats 100 hrs post-treatment with 20% exhibiting light-yellow patches throughout the myocardium. The patches consisted of necrotic cardiac muscle, partly infiltrated by polymorphonuclear leukocytes and monocytes. These myocardial lesions were induced in all nephrocalcinotic rats subjected to neuromuscular stress for 3-4 days. Neither nephrocalcinosis nor the myocardial lesions could be observed when the steroid was administered alone.

3. Ref. 920 (sodium pyrophosphate)

The action of sodium pyrophosphate (NaPP), as a physiological regulator of calcification on female Sprague-Dawley rats, was studied.

A calcified wheal was caused within 6 days when 100 gm rats received s.c. 150 μg in 0.2 ml H_2O of either $FeCl_2$ or $CrCl_2$ after pretreatment with 1 mg dihydrotachysterol (DHT) by stomach tube. This calcification was prevented by simultaneous s.c. injection of NaPP (0.21 mg P in 0.1 ml) with the metal salts.

If 200 μg in 0.2 ml H_2O of either $HoCl_3$ or $PbCl_2$ were injected s.c. between the shoulders of 100 gm rats the calcified wheal could again be observed in 6 days. This wheal was prevented by simultaneous injection of NaPP (0.14 mg P) s.c. at the same spot as the metal salts. The typical crystal pattern of hydroxyapatite was found upon X-ray examination of calcified deposits.

In another study i.v. injection of NaPP (1.2 mg P in 1 ml), but not an equivalent amount of orthophosphate, caused a transient hypercalcemia in 200 gm rats. This disappeared within 1 hour. NaPP also reduced the effects of calcium overdosage in 200 gm rats. Administration of 2 mmoles of calcium acetate in 2 ml H_2O by stomach tube caused hypercalcemia and hypercalcuria, observed at 90 min. after treatment. When NaPP (1.2 mg P in 1 ml H_2O) was given i.v. 30 min after calcium treatment, analysis of blood and urine at 90 min showed Ca levels had returned to normal.

When 200 gm rats received either 2.5 mg (DHT) in 0.5 ml corn oil by stomach tube or 150 I.U. of parathyroid extract twice in two days s.c., blood and urine calcium were significantly increased at 24 hours after DHT treatment and immediately after the last parathyroid extract injection. Injections s.c. of NaPP (1.2 mg P), at 22 or 46 min after DHT administration or the first parathyroid extract injection, partially counteracted the hypercalcemic effects of both. It partially counteracted the hypercalcuric effect of DHT but not of parathyroid extract and it did not modify calcification of the myocardium, aorta or proximal convoluted tubules of the kidney caused by DHT or parathyroid extract, found after sacrifice on the 6th day.

4. Ref. 322 (NaH_2PO_4)

For 12 days groups of 110 - 330 gm male and female albino rats were given 120 mg NaH_2PO_4, twice daily, by gavage. In some groups daily i.m. injection of 1.2 mg/kg of hydrocortisone (HCS) and/or 1.2 mg/kg of deoxycortisone (DOC) were administered concurrently. At the end of 12 days the animals were sacrificed and the brain, heart and adrenal glands were removed, weighed and analyzed for catecholamines.

Administration of sodium phosphate, alone, caused a significant increase in noradrenaline concentration in the heart, brain and adrenal glands while adrenaline concentration was unaffected. The average weight of the adrenal glands was lower in animals receiving this treatment than in controls. When this experiment was performed again, however, sodium phosphate treatment did not affect catecholamine concentration of the organs analyzed or adrenal gland weight.

After combined sodium phosphate and HCS treatment catecholamine concentration did not differ from untreated controls but the average weight of the adrenal glands was 11% higher. Combined sodium phosphate and

DOC treatment promoted higher noradrenaline concentration only in the adrenal glands and adrenaline concentration and the weight of the organs examined were unaffected. When all three were administered simultaneously the only noticeable change was a lower adrenal gland weight than in untreated controls.

5. Ref. 818 (sodium orthophosphate)

For 12 days 100 gm female Holtzman rats were given 750 μg of fluorocortisol acetate daily s.c. in 0.2 ml H_2O and NaH_2PO_4 or Na_2HPO_4 (320 mg P/kg) in H_2O twice daily by gavage, alone or conjointly with 1 ml corn oil by stomach tube. Without oil supplements fluorocortisol-phosphate treatment failed to produce electrolyte-steroid cardiopathy with necrosis and caused a 0 - 10% mortality. In contrast, in the rats also receiving corn oil, severe cardiac necroses and a high mortality (80 - 90%) were elicited by the steroid-phosphate treatment.

6. Ref. 426 (NaH_2PO_4)

The percentage of fat absorbed in 4 hr by 6 48 - hr fasted adult rats fed 1.5 ml of a melted hydrogenated vegetable shortening followed by 1 ml of a 2 mg NaH_2PO_4 solution by gavage was 47%. This was 7% lower than that for 11 control rats. Based on independent assay with different concentrations of various food additives, including sodium glycerophosphate, it was concluded that hydrotropic agents fail to potentiate fat absorption rates at low doses, while higher concentrations are inhibitory.

V. Consumer Exposure Information

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

Possible daily intake in 2 - 65 age group in mg P

Food Category	Average*	High A**	High B***
Baked goods	925	1334	992
Breakfast cereals	114	292	149
Frozen dairy products	7	18	6
Instant coffee, tea	36	77	41
Meat products	93	131	138
Poultry	36	89	60
Processed vegetables	181	305	199
Fats, oils	30	56	32
Cheese	74	189	153
Gravies	19	47	20
Milk products	138	421	419
Gelatin, pudding	65	166	82
Soups	13	35	17
Sweet sauce	10	24	10
Non-alcholic beverages (except milk, fruit)	26	68	61
Total	1767	3252	2379

Possible daily intake (mg. P) in 0 - 5 and 6 - 11 month age group.

Food Category		Average*	High A**	High B***
Formulas	0 - 5 mo.	840	1536	961
	6 - 11 mo.	169	799	195
Baby Cereals	0 - 5 mo.	46	80	25
	6 - 11 mo.	31	83	33

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

A 60 kg man consuming 1500 gm of solid food per day could consume 6.7 gm of phosphate if the maximum amount of additive, reported in the NAS/NRC survey, were used in the following extreme diet.

Substance	Amount	gm P*
Cheese (3.1% Na_2HPO_4)	300 gm	2.0
Milk products (3.5% Na_2HPO_4)	500 gm	3.8
Breakfast cereals (1.29% $CaHPO_4$)	200 gm	0.46
Reconstituted vegetables (0.96% $CaHPO_4$)	200 gm	0.34
Baked goods (1.04% $Na_2H_2P_2O_7$)	100 gm	0.14
Other	200 gm	
	Total	6.7 gm

* There is also a low level of phosphate occurring naturally in all biological samples (see Occurrence section), not included in the calculation.

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APPENDIX B

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

to

PHOSPHATES

A Monograph

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

by

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Dr. Jon E. Villaume and
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FIRL Project No. 80G-C3378-01
January 26, 1973

Fd Cosmet. Toxicol. Vol. 2, pp. 147–154. Pergamon Press 1964. Printed in Great Britain

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SUMMARIES OF TOXICOLOGICAL DATA

TOXICITY STUDIES ON PHOSPHATES

Summaries of four unpublished reports of work carried out on behalf of Calgon Company by Professor Harold C. Hodge and colleagues from the Division of Pharmacology and Toxicology, University of Rochester School of Medicine and Dentistry, Rochester, New York.*

I. Short-term oral toxicity tests of condensed phosphates in rats and dogs

(*Report dated* 19 *October* 1956)

Introduction

The customary purpose of a short-term test of oral toxicity carried out on small groups of animals is to prepare the ground for chronic studies; and in particular to permit the selection of suitable concentrations of the test substance. In the present instance a more elaborate investigation was undertaken because of the reported harmful effects of large amounts of orthophosphates on the kidney. Consequently the studies summarized below were directed primarily towards the discovery and assessment of renal damage.

The materials used were: sodium hexametaphosphate, Curafos URS (HMP): sodium tripolyphosphate, Curafos (TPP); sodium trimetaphosphate (TMP); and sodium tetrametaphosphate (TetMP).

Feeding test in rats

Groups of 5 male weanling rats were maintained for 1 month on diets composed of Fox Chow meal with the following additions: group I, controls—basal ration only; group II, controls—10% sodium chloride; group III, controls—5% sodium orthophosphate, (OP); groups IV (a,b,c), 0·2, 2·0 and 10% respectively of HMP; groups V (a,b,c), VI (a,b,c) and VII (a,b,c) correspondingly for TPP, TMP and TetMP. It is stated that enough calcium was added to each diet to ensure a "reasonably balanced" ratio of calcium to phosphorus.

Growth. Retardation of growth was observed in rats of group II and in all groups (c) on diets containing 10% condensed phosphates.

Kidney weights. Rats were killed on days 3, 7, 15 and 28. In animals of group IV(c) the kidneys were pale and swollen. Increases in kidney weight relative to body weight were observed in groups V(c), IV(c), VII(c) and II (in decreasing order of magnitude).

Histopathology of kidneys

5 *and* 10% *Phosphate diets.* Tubular necrosis was present in the kidneys of rats in group III and in groups IV(c)–VII(c); groups IV(c) and V(c) were particularly affected. TMP [group VI(c)] produced a definite effect by day 15 but it was apparently transient for by day 28 no deviation from normality could be detected. There was some suggestion of a similar effect by OP (group III).

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Food and Cosmetics Technology. 2(1), 1964, pp. 147–154

2% *Phosphate diets*. A number of rats exhibited acute inflammatory changes of the renal pelvis on day 28. In 3 rats there were tubular lesions, possibly ascribable to the action of condensed phosphates.

0·2% *Phosphate diets*. The kidneys of these rats were unaffected by the special diets.

Feeding test in dogs

In a low-level feeding test conducted over a period of 1 month, 4 young adult dogs were given diets providing 0·1 g/kg/day of each of the condensed phosphates, HMP, TPP, TMP and TetMP. In a high level feeding test a further group of 4 dogs was fed for 5 months on diets containing the 4 phosphates, starting with a dose of 1 g/kg which was increased finally to 4 g/kg.

Growth. Small weight losses which were believed not to be significant were observed in the 2 dogs fed HMP and TetMP, and weight losses, which occurred mainly after the dose had been increased to 4 g/kg, were observed in each of the dogs in the high level test.

Haematology. Blood samples examined at the start and at the end of the tests gave haematological values in the normal ranges, except for some elevation of eosinophil counts and a decrease in the proportion of neutrophils in 3 of the dogs in the high level test.

Urine analysis. Only traces of reducing substances and protein were found.

Organ weights. Dogs in the high level test yielded increased heart weights, but heart weights were unaffected in the other dogs. Kidney weights were normal.

Histopathology. The kidneys of all dogs given condensed phosphates at the high dosage level displayed tubular damage similar to the phosphate nephritis found previously in the rats. Other tissues were in general normal, except for hypertrophy of the left ventricle in the hearts of dogs used in the high level tests. At doses of 0·1 g/kg/day no significant effect was observed in any organ.

II. Chronic oral toxicity studies of sodium tripolyphosphate in rats

(*Report dated* 3 *October* 1959)

Preliminary studies

Rats. The acute LD_{50} of sodium tripolyphosphate (TPP) was 525±52 mg/kg. Groups of 14 male rats were maintained for 1 month on diets composed of Fox Chow meal with the following additions: group I, control—basal ration only; groups II, III and IV received 0·2, 2·0 and 10% respectively.

Slight retardation of growth was observed in rats in groups II and III and marked retardation in group IV. Kidney weights were possibly slightly elevated in group III and substantially raised in group IV. Tubular degeneration and necrosis of the kidneys were observed in rats of group IV.

Dogs. One dog fed 0·1 g TPP/kg/day for 1 month grew well and exhibited normal tissues on histological examination.

A second dog maintained for 154 days on a diet containing 1 g TPP/kg/day increasing to 2·5 g/kg/day by the end of the test, exhibited "phosphate nephritis" and left ventricular hypertrophy. Other organs and blood and urine analyses revealed no abnormality.

Chronic feeding studies

Groups of 50 weanling rats of both sexes of the Rochester strain were maintained for 2 yr

on diets composed of Fox Chow meal with the following additions: group I, control—basal diet only; groups II, III and IV received 0·05, 0·5 and 5·0% TPP respectively.

Growth and food consumption. Rats in groups I, II and III showed comparable growth during the 2-yr period. Growth of male rats in group IV was clearly retarded throughout the test period. Females in group IV showed slight growth retardation during the first yr which became more obvious during the second yr. Food intake measured at 1 and 3 months was greater in rats of group IV than those of group I controls.

Haematology. At 1 yr there was evidence of anaemia in male rats of group IV. The red blood cells counts, haemoglobin values and haematocrits were somewhat lower than those observed in the other groups. Otherwise the haematological indices were within normal limits.

Urine analysis. Only traces of protein and reducing substances were found.

Mortality. Mortality was high, principally because of epidemics which occurred at various times. The highest mortality (80%) over the 2-yr period was observed in females of group IV and the lowest in females of group II. During the second yr there was heavy mortality in both males and females of group IV. No relation was apparent between dose and mortality in rats of groups II and III. Respiratory infection and pericarditis–peritonitis were the most prominent causes of death.

Organ weights. Kidney weights were greatly increased in male rats of group IV. There were also slight increases in the weights of liver, testes, brain, stomach and heart, possibly attributable to the smaller body weights. In some instances increases in liver and kidney weights relative to body weight were observed in female rats of group IV.

Bone analyses. Femur lengths and body weights in males and females of group IV were smaller than those of control animals, indicating a true reduction in growth. The femurs of both sexes in this group contained slightly more water and slightly less organic matter as compared with controls. The ash contents and ratios of calcium to phosphorus were normal for both sexes. Radiographically the bones of rats of groups II, III and IV appeared normal.

Histopathology. Striking changes occurred in the kidneys of all rats in group IV, the most important being enlargement of this organ, associated with dilated convoluted tubules and hyaline casts. Accompanying these changes were interstitial fibrosis between the dilated tubules and fibrotic glomeruli, as well as intertubular calcification. To this lesion the term 'chronic tubular nephropathy' was applied. It had to be distinguished from the coexistent chronic lesion present in old rats ('chronic pyelonephritis'). In control rats and those of groups II and III the kidney changes were indistinguishable from chronic pyelonephritis of old rats. It seems a fair assumption therefore that the lesion attributable to TPP was limited to the 5% group IV.

There was no indication that TPP is carcinogenic. The tumours observed were of the types usually found in old rats, and their incidence was the same in all groups.

Reproduction studies

Reproduction studies covered 3 generations of rats reared and maintained on diets containing 0 and 0·5% TPP. Two litters were examined in each generation. Matings were carried out between 16 females and 8 males of each group when the rats were 100 days old.

TPP had no effect on fertility or litter size, nor on growth or survival of offspring. Organ weights of third-generation rats sacrificed at 1 month were unaffected. A slight increase in

kidney weight was observed but was not statistically significant. The macroscopic and microscopic appearances of major organs in third-generation rats were comparable in both groups.

III. Chronic oral toxity studies of sodium trimetaphosphate in rats

(*Report dated* 20 *January* 1960)

Preliminary studies

Rats. The acute LD_{50} of sodium trimetaphosphate (TMP) when given intraperitoneally to female rats was 3650±620 mg/kg. Groups of 5 male rats were maintained for 1 month on diets composed of Fox Chow meal with the following additions: group I, controls—basal ration only; group II, controls—10% sodium chloride; group III, controls—5% disodium phosphate; groups IV (a,b,c), 0·2, 2·0 and 10% respectively of TMP.

Growth of rats in groups I, III, IV(a) and IV(b) was comparable but was retarded in rats of groups II and IV(c). Rats in group IV(c) displayed slightly increased kidney weights and only slight transient changes in the kidney tubules ('transient' in the sense that they were present at 15 days but not at 28 days). Kidneys of rats in group II were heavier than normal and displayed kidney changes, but of a different sort. Tubular and other pathological effects were seen in the kidneys of rats in group III.

Dogs. One dog fed 0·1 g/kg/day for 1 month maintained normal body weight. Urine analysis, haematological tests and histological examinations of tissues showed no abnormality. Organ weights were normal.

A second dog fed doses increasing from 1–4 g/kg/day for the same period lost 1·4 kg in body weight. Its heart weight lay in the upper limit of the normal range of heart weights. Renal tubular damage was present similar to the phosphate nephritis observed in rats.

Chronic feeding studies

Groups of 50 weanling rats of both sexes were maintained for 2 yr on diets composed of Fox Chow meal with the following additions: group I, controls—basal ration only; groups II, III and IV received 0·1, 1·0 and 10·0% TMP respectively.

Growth and food consumption. Rats in groups I and II grew normally. Males in group III showed slight growth retardation up to the age of 6 months after which retardation became more pronounced; however growth rose to normal during the second yr. There was no growth retardation in the females of this group. Substantial reduction in growth was observed in males and females of group IV. Food consumption by rats in this group was normal at 1 and 4 months. The observed reduction in growth rate might in part be attributed to a cathartic action of TMP.

Haematology. All indices were in the normal range except for lower red cell counts and haematocrits in female rats of group IV.

Urine analysis. Only traces of protein and reducing substances were found.

Mortality. The highest mortality was observed in the females of group IV (88%). There was a 58% mortality of females and 70% of males in group II. Except in the females in group IV, lower levels of dietary TMP did not appear to influence mortality. The principal causes of death were respiratory infection or pericarditis–peritonitis.

Organ weights. The organs in all groups except group IV had comparable weights. Owing

to the growth impairment in group IV, some organs were heavier on a body weight basis. The liver was a striking exception, being considerably smaller than in other groups, both in males and females of group IV.

Bone analyses. Femur lengths of males in group IV were shorter than those in rats of other groups but in view of the decreased body weights of these animals the ratio of femur length to body weight was comparable in all groups. Femurs were also shorter in females of group IV, but when body weights were taken into account, the ratios were higher than in those obtained with other groups. There was thus a true retardation of growth.

Although the ratio of calcium to phosphorus was normal in all rats, the levels of calcium and phosphorus were elevated in males and females of group IV. Males in group IV showed less water and more ash. On the other hand more water and less ash were present in bones of females in this group.

Histopathology. No changes were found that could be attributed to the administration of TMP. Concretions were present in the kidneys of some rats, particularly females, in groups I, II and III. Calcification, believed to have followed infection by a nematode, was observed in the collecting tubules of the kidneys of group IV rats. The overall yield of tumours was low. There was no correlation between the dietary level of TMP and tumour incidence.

Reproduction studies

Reproduction studies covered 3 generations of rats reared and maintained on diets containing 0 and 0·05% TMP. Two litters were examined in each generation. Matings were carried out between 16 females and 8 males of each group when the rats were 100 days old.

TMP had no effect on fertility or litter size, nor in growth or survival of offspring. No abnormality in the organ weights of third-generation rats at weaning were evident. Histologically, tissues of third-generation rats appeared normal.

IV. Chronic oral toxicity studies of sodium hexametaphosphate in rats

(*Report dated* 16 *April* 1960)

Preliminary studies

Rats. The acute oral LD_{50} of sodium hexametaphosphate (HMP) in female rats was 2900±258 mg/kg. Groups of 5 male rats were maintained for 1 month on diets composed of Fox Chow meal with the following additions: group I, controls—basal ration only; group II, controls—10% sodium chloride; group III, controls—5% disodium orthophosphate; groups IV (a,b,c), 0·2, 2·0 and 10% respectively of HMP.

Retardation of growth was observed in rats of groups II, IV(a) and IV(c). Normal growth was maintained in rats of groups I, III and IV(b). Growth inhibition of rats in group IV(a) could not be explained. Rats in group IV(c) had increased kidney weights and suffered kidney injury characterized by tubular degeneration and necrosis ("phosphate nephritis"). Some lesions of the same type were observed in group IV(b) but none in group IV(a).

Dogs. One dog fed 0·1 g HMP/kg/day for 1 month lost an insignificant amount of weight. Urine and blood analyses and organ weights were not affected by HMP. No histological abnormalities were evident.

A second dog maintained for 154 days on a diet initially containing 1 g/kg/day, increasing to 4 g/kg/day for 1 month lost weight significantly. Increased heart weight with left ventricular hypertrophy and kidney changes ("phosphate nephritis") were the other abnormal findings.

Chronic feeding studies

Groups of 50 weanling rats of both sexes were maintained for 2 yr on diets composed of Fox Chow meal with the following additions: group I, controls—basal ration only; groups II, III and IV received 0·05, 0·5 and 5·0% HMP respectively.

Growth and food consumption. No effect on growth was observed in groups II and III. In group IV, growth inhibition was obvious in males but females were only slightly affected. Food consumption measured at 90 and 210 days was greater in rats of group IV than those of group I. Interference with food absorption or assimilation probably accounted for the reduced growth in rats of group IV. These data were consistent with a mild cathartic action which could carry the food through the gastro-intestinal tract more rapidly and consequently lead to a slight increase in food intake.

Haematology. Periodical examination of blood revealed no abnormality.

Urine analysis. Only traces of protein and reducing substances were found.

Mortality. There was no indication of an increase in mortality with increasing level of dietary HMP. An apparent slight increase in females was considered insignificant. Over the 2-yr period mortality ranged from 64–78%, with females surviving better than males. The median lifespan was highest in males of group III. The most common cause of death was respiratory infection.

Organ weights. Increased kidney to body weight ratios were found in rats of both sexes of groups III and IV. Organ weights were difficult to interpret owing to the varied incidence of kidney infection, which was higher among HMP-fed rats than controls. There were also possibly increased lung weights and reduced spleen weights in females of group IV.

Bone analyses. Femur lengths were unaffected; reduction in femur lengths of males in group IV was not considered significant. Femurs from all rats had comparable water, ash, calcium and phosphorus contents. Radiographically, the femurs appeared normal.

Histopathology. None of the rats in groups I and II, only 1 female rat in group III, and 13 out of 20 rats in group IV exhibited calcification in the tubules of the kidneys. The calcification, which was the only pathological change attributable to the administration of HMP, was believed to represent an effect superimposed on the normal processes of infection and degeneration in kidneys of old rats. Kidney lesions in rats fed HMP were less severe and occurred to a lesser extent than those seen in rats fed TPP. A few more tumours were found in control rats as compared with rats fed HMP. Tumour incidence could not be correlated with increasing levels of HMP and there was no evidence to suggest that HMP was carcinogenic.

Reproduction studies

Reproduction studies covered 3 generations of rats reared and maintained on diets containing 0 and 0·5% HMP. Two litters were examined in each generation. Matings were carried out between 16 females and 8 males in each group when the rats were 100 days old.

HMP had no effect on fertility or litter size, nor on growth or survival of offspring. In third-generation rats no abnormal organ weights or histological changes were evident.

Overall conclusions

These may be brought together from the summary table as follows:

The 'no-effect' level of the four condensed phosphates when incorporated in the diet of rats over a period of 1 month was 2% in all cases. Extension of the feeding tests for 24 months indicated an absence of significant toxic effect at levels intermediate between 0·05 and 0·5% in the case of HMP, 0·5 and 5·0% in the case of TPP and 0·1 and 1% for TMP.

In dogs daily doses of 0·1 g condensed phosphates/kg body weight for 1 month brought about no adverse effect. Feeding at high levels over a period of 5 months resulted in poor growth, increased heart weights and injury to heart and kidney.

Table 1. *Summary of short-term and chronic toxicity studies on condensed phosphates in rats*

NEO = No effect observed M = Males F = Females

Compound and acute toxicity in females (LD_{50} mg/kg)	Dietary level (%)	Duration of feeding (months)	Growth reduction	Haematology	Organ weights: Kidney	Organ weights: Other organs	Femur length and analyses	Renal micropathology T = tubules
HMP 2900 (oral)	0·05	24	None	NEO	NEO	NEO	NEO	NEO
	0·20	1	Slight (unknown cause)					NEO
	0·50	24	None	NEO	Increase	NEO	NEO	T—increased calcification (in 1 F)
	2·0	1	None		NEO			T—minimal necrosis
	5·0	24	Some (M); slight (F)	NEO	Large increase (F); increase (M)	Possible increase in lungs and decrease in spleen (F)	NEO	T—increased calcification
	10·0	1	Some		Increase			T—degeneration and necrosis ("phosphate nephritis")
TPP 525 (route unspecified)	0·05	24	None	NEO	NEO	NEO		NEO
	0·20	1	Slight					NEO
	0·50	24	None	NEO	NEO	NEO		NEO
	2·0	1	Slight		Possible slight increase			T—minimal necrosis
	5·0	24	Some (M); slight (F)	Anaemia (M); possibly (F)	Increase (M); increase (F, body wt ratio)	Slight increase in liver and 4 other organs (especially M)	Shorter femurs; H_2O raised, organic matter reduced	"Chronic tubular nephropathy"
	10·0	1	Substantial		Large increase			T—degeneration and necrosis

NEO = No effect observed M = Males F = Females

Compound and acute toxicity in females (LD_{50} mg/kg)	Dietary level (%)	Duration of feeding (months)	Growth reduction	Haematology	Organ weights: Kidney	Organ weights: Other organs	Femur length and analyses	Renal micropathology T = tubules
TMP	0·10	24	None		NEO	NEO		Concretions (F, as in F controls)
3650 (i.p.)	0·20	1	None		NEO			NEO
	1·0	24	Slight (M, 1st yr only)		NEO	Decrease liver wt (F)		Concretions (F, as in F controls)
	2·0	1	None		NEO			T—minimal necrosis
	10·0	24	Substantial	Anaemia (F)	Increase (body wt ratio)	Decrease in liver; increase in other organ to body wt ratios	Shorter femurs; raised Ca and P levels (H_2O reduced, ash raised M, converse for F)	T—increased calcification
	10·0	1	Some		Slight increase			Slight transient "phosphate nephritis"
TetMP	0·20	1	None					NEO
	2·0	1	None		NEO			NEO
	10·0	1	Some		Increase			Slight "phosphate nephritis"

Mortality, reproduction performance covering 3 generations at dietary levels of 0·05% for TMP and 0·5% for HMP and TPP, and urine analysis have been omitted because the differences observed between test and control groups were not considered significant.

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Quart. J. Florida Acad. Sci. 25: 161-6 (1962)

ABSORPTION AND TISSUE DISTRIBUTION OF P^{32} FROM DIFFERENT INORGANIC SOURCES IN RATS[1, 2]

L. R. Arrington, J. C. Outler[3], C. B. Ammerman and G. K. Davis
University of Florida

The degree of absorption of phosphorus from various inorganic sources is of importance in supplying phosphorus to livestock and poultry. Several different phosphate compounds are used and their value depends in part upon the biological availability of the phosphorus present. Methods which have been used to measure availability have generally been evaluations based upon growth, bone ash, bone and blood phosphorus, phosphorus balance and retention, and utilization by micro-organisms. Labeled phosphorus in solution has been used in many experiments to study absorption and tissue accumulation of the element (Cohn and Greenberg, '38; Manly and Bale, '59; Feaster, *et al.*, '53; Arrington and Davis, '55). Few studies have been reported in which labeled phosphorus in compounds, not in solution, were evaluated. Davis, *et al.* ('53) and Arrington, *et al.* ('59) have reported values for the absorption of labeled phosphorus in compounds which were administered orally to cattle and swine. Lofgreen ('60) employed an isotope dilution technique in studying the availability (true digestibility) of different phosphate compounds for sheep.

The objective of this study was to determine the absorption and tissue deposition of P^{32} from dicalcium phosphate, defluorinated rock phosphate, soft phosphate and phosphoric acid as a measure of the relative biological availability.

Procedure

Samples of reagent grade dicalcium phosphate, defluorinated rock phosphate and soft phosphate with colloidal clay were irradiated in the pile at Oak Ridge National Laboratory to convert a portion of the P^{31} to P^{32}. Defluorinated rock phosphate and soft phosphate were produced in Florida and samples were obtained by the authors at mining areas. Following the two-week service

[1] Florida Agricultural Experiment Station, Journal Series No. 1456.
[2] Supported in part by grants from Smith-Douglass Company, Norfolk, Va. and Soft Phosphate Research Institute, Ocala, Fla.
[3] Present Address: Sperry Corporation, Gainesville, Fla.

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irradiation, the labeled compounds were administered as a single oral dose in the dry form to growing rats. The dosage for each rat amounted to 20-50 mg and was adjusted to provide approximately 5 μc of activity. The compounds were administered with a stomach tube which was flushed with 2 ml of distilled water. A solution of P^{32} labeled phosphoric acid in weak HCl was also given orally to eight rats. One-half ml of the solution provided the same activity as that administered in the other forms. Eight additional rats were given a single subcutaneous dose of the P^{32} solution which had been diluted with physiological saline to provide the same activity administered orally to the other experimental rats.

The rats were of the Long-Evans strain, 9 to 11 weeks of age. The composition of the diet in percent was ground yellow corn, 78; gluten, 18; Brewer's yeast, 2; NaCl, 1; and $CaCO_3$, 0.6; and contained 0.34% of calcium and 0.36% of phosphorus. Following administration of the labeled phosphates, rats were confined to metabolism cages for quantitative collection of feces and urine. Tissue distribution studies were made on selected tissues removed from rats sacrificed as follows: phosphoric acid, 8 rats at 72 hours; subcutaneous P^{32}, 8 rats at 72 hours; dicalcium phosphate, 6 rats at 12 hours, 6 at 24, 3 at 48 and 3 at 72 hours; defluorinated rock phosphate, 5 at 12 hours, 5 at 24, 4 at 48 and 5 at 72 hours. Accumulation of P^{32} in the tissues of rats given the soft phosphate was not studied. Measurement of the deposition in the tissues of rats given dicalcium phosphate and defluorinated rock phosphate at the time intervals shown was undertaken in order to observe the rate of P^{32} deposition.

The urine was diluted with weak HCl for radioactivity measurement. Feces and tissues were wet digested in nitric acid and radioactivity determined with a commercial GM dipping tube and scaler. The amount of labeled phosphorus excreted in the feces and urine was calculated as the percent of administered dose. Tissue accumulation was calculated on the basis of percent of dose per gram of fresh tissue. Statistical calculations were based on the analysis of variance according to Snedecor, '56.

Results and Discussion

The cumulative fecal and urinary excretion of P^{32} is shown in Figure 1. Rats which had received the P^{32} solution orally excreted significantly less ($P<0.01$) of the labeled phosphorus in the

feces than did any of the
phate compounds. These
gesting a greater retention
values are slightly higher, b
reported by Arrington and
portion ($P<0.05$) of the re
excreted in the feces than o
phosphate. If the difference
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phorus as phosphoric acid
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rock phosphate. Absorption
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Statistical calculations we
tion at 48 hours. Approxima
present in the feces at this ti
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cretion by 48 hours was 92%

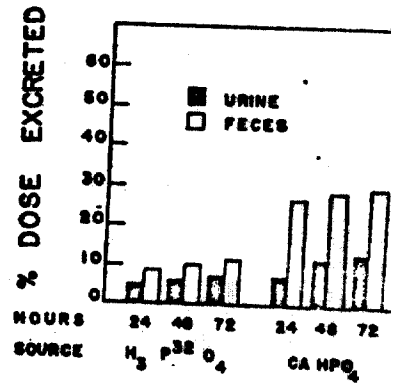

Fig. 1. Cumulative urinary and
phosph

Elimination of the labeled
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than through the gut (Figure 1
only 1.5% of the dose had been
5.5% in the urine. This is a ra

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9 to 11 weeks of age.
round yellow corn, 78;
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retion of P^{32} is shown
P^{32} solution orally ex-
eled phosphorus in the

feces than did any of the other groups receiving the other phosphate compounds. These rats also excreted less in the urine suggesting a greater retention as well as greater absorption. These values are slightly higher, but are of the same order as those earlier reported by Arrington and Davis (55). A significantly smaller proportion ($P<0.05$) of the reagent grade dicalcium phosphate was excreted in the feces than of defluorinated rock phosphate or soft phosphate. If the difference between the total dose administered and total fecal P^{32} is used as a measure of absorption, the phosphorus as phosphoric acid was absorbed to the greatest degree followed by dicalcium phosphate, soft phosphate and defluorinated rock phosphate. Absorption values for soft phosphate and defluorinated rock phosphate were not significantly different. The values represent apparent absorption since no correction for endogenous P^{32} was applied.

Statistical calculations were based upon the cumulative excretion at 48 hours. Approximately 98% as much of the isotope was present in the feces at this time as at 72 hours, and a greater number of values were available for the 48 hour period. Urinary excretion by 48 hours was 92% as much as at 72 hours.

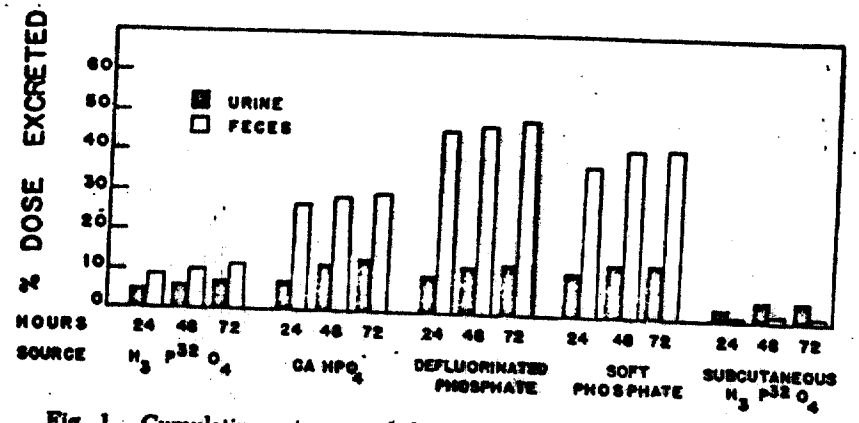

Fig. 1. Cumulative urinary and fecal excretion of P^{32} as affected by phosphorus source.

Elimination of the labeled phosphorus which was administered subcutaneously occurred to a greater degree through the kidney than through the gut (Figure 1). At 72 hours after administration only 1.5% of the dose had been excreted in the feces compared to 5.5% in the urine. This is a ratio of approximately 3.5:1. At 24

hours after administration 61% as much as at 72 hours had been eliminated in the urine. During the same time 45% as much as at 72 hours had been eliminated in the feces. Different relative rates of excretion were also observed between the two methods of administration. At 24 and 48 hours the fecal excretion of P^{32} given orally was 76.7 and 96.4% as much as at 72 hours, respectively. At the same time intervals, excretion from the subcutaneous doses were 45.4 and 81.8% as much as at 72 hours.

Tissue deposition of the labeled phosphorus is shown in Figure 2 as percent of dose per gram of fresh tissue at 72 hours after administration. Relative absorption values indicated in Figure 1 are reflected in the tissue accumulation of the labeled phosphates from the respective compounds. The proportion of orally administered P^{32} in all tissues from the solution was numerically greater than for the other two phosphate compounds and was significantly greater for kidney and muscle ($P<0.05$) and femur ($P<0.01$). Concentration of P^{32} in all tissues except femur was greater in rats which had received dicalcium phosphate than in tissues of rats which had received defluorinated rock phosphate.

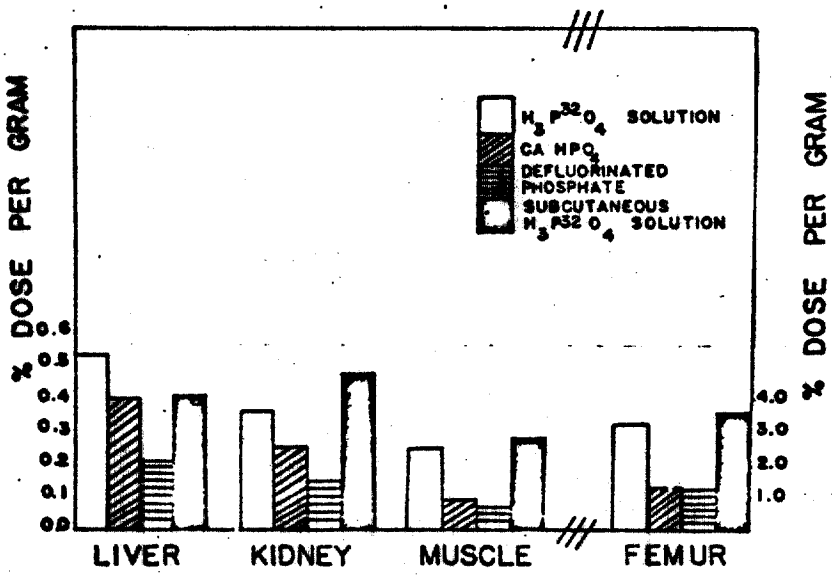

Fig. 2. Tissue deposition of P^{32} from different phosphate sources.

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TISSUE ACCUMULATION O
PHOSPHATE AND DE

Tissue	Source
Blood	Ca H PO_4
	Defluorinated P*
Liver	Ca H PO_4
	Defluorinated P
Kidney	Ca H PO_4
	Defluorinated P
Muscle	Ca H PO_4
	Defluorinated P
Femur	Ca H PO_4
	Defluorinated P

* Defluorinated rock phosphate.

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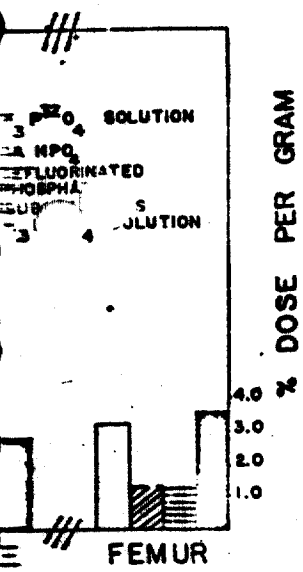

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Labeled phosphorus in the blood at 72 hours from all sources was less than one tenth percent of the dose, but the amounts represented the same relative pattern of accumulation.

Rats which had received dicalcium phosphate and defluorinated phosphate were sacrificed at intervals of 12, 24, 48 and 72 hours and measurements of P^{32} in the tissues were made in order to observe the relative rates of deposition. The results for 12 to 48 hours are recorded in Table 1 and for 72 hours in Figure 2. The amounts present at 12 and 24 hours were of the same order with a decrease to 72 hours. The greatest proportional decrease occurred between 24 and 48 hours.

TABLE 1

TISSUE ACCUMULATION OF PHOSPHORUS-32 FROM DICALCIUM PHOSPHATE AND DEFLUORINATED ROCK PHOSPHATE.

Tissue	Source	Hours 12	24	48
		% dose per gram fresh tissue		
Blood	Ca H PO_4	0.175	0.169	0.058
	Defluorinated P*	0.081	0.066	0.028
Liver	Ca H PO_4	1.345	1.155	0.314
	Defluorinated P	0.515	0.513	0.274
Kidney	Ca H PO_4	0.856	0.835	0.288
	Defluorinated P	0.274	0.267	0.188
Muscle	Ca H PO_4	0.215	0.264	0.084
	Defluorinated P	0.080	0.085	0.056
Femur	Ca H PO_4	2.920	2.172	1.529
	Defluorinated P	1.320	1.630	0.650

* Defluorinated rock phosphate.

Summary

Samples of dicalcium phosphate, defluorinated rock phosphate and soft phosphate were irradiated and administered orally to rats in order to determine relative absorption and tissue deposition of the labeled phosphorus. Radioactive phosphorus in solution as H_3PO_4 was administered orally and subcutaneously to additional

rats. The P^{32} as phosphoric acid was absorbed in the largest amount and deposited in greatest proportion in the tissues. The proportion of dicalcium phosphate absorbed was greater than either defluorinated phosphate or soft phosphate but less than P^{32} in solution. No significant differences were observed in the absorption of defluorinated phosphate and soft phosphate. Phosphorus-32 administered subcutaneously to rats was excreted largely through the kidney.

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КРАТКИЕ СООБЩЕНИЯ

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СПЕЦИФИЧНОСТЬ И ОБРАТИМОСТЬ СТАБИЛИЗАЦИИ СПЕРМАТОЗОИДОВ НЕКОТОРЫМИ ГИСТОНАМИ

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Очень богатый лизином гистон тимуса теленка (*f1*) стабилизирует сперматозоиды хряка по тесту подвижности. Эффект специфичен: все остальные гистоны тимуса (*f2a*, *f2в* и *f3*), протамин и путресцин подобным действием не обладают. Напротив, богатые аргинином гистоны *f2a* и *f3* подавляют активность спермиев. Действие гистона *f1*, связавшегося со спермиями, полностью снимается метафосфатом натрия в течение первых 30 *мин*. Через 45 *мин* взаимодействия эффект становится необратимым.

Изучение влияния основных белков и диаминов на стабильность сперматозоидов имеет не только практическое, но и существенное теоретическое значение. Все эти факторы известны как агенты, участвующие в стабилизации третичной структуры нуклеиновых кислот в условиях особенно плотной их упаковки *in vivo* (фаги, животные вирусы, спермии и др.) и *in vitro*. Основные белки сравнительно легко проникают через клеточные мембраны и оказывают значительное влияние на проницаемость этих мембран для других веществ [1, 2]. Широко изучено подавляющее действие гистонов на транскрипцию ДНК [3]. Однако эту их способность вряд ли следует учитывать, рассматривая их действие на спермии, так как ДНК последних либо не транскрибируется вовсе, либо процесс этот очень ограничен [4—7]. Наконец, гистоны оказывают сложное (в некоторых случаях активирующее, а чаще подавляющее) действие на активность ряда энзиматических систем [3, 8, 9].

Все это побудило нас к изучению влияния гистонов на переживаемость сперматозоидов. При этом выявилось существенное стабилизирующее действие гистонов тимуса, богатых лизином [10]. Стремясь установить механизмы этого явления, мы в настоящей работе пошли по пути оценки специфичности эффекта, сравнивая его с действием других основных белков и диаминов, а также по пути изучения стадий процесса стабилизации.

Гистоны получали из тимуса одномесячных телят вторым методом Джонса [11]. Некоторые особенности полученных при этом препаратов описаны одним из авторов ранее [12]. Использовали также коммерческие препараты протамина и путресцина (1,4-диаминобутан дихлоргидрида). Объектом исследования были сперматозоиды хряков. Условия содержания и кормления животных, режим взятия семени, а также мето-

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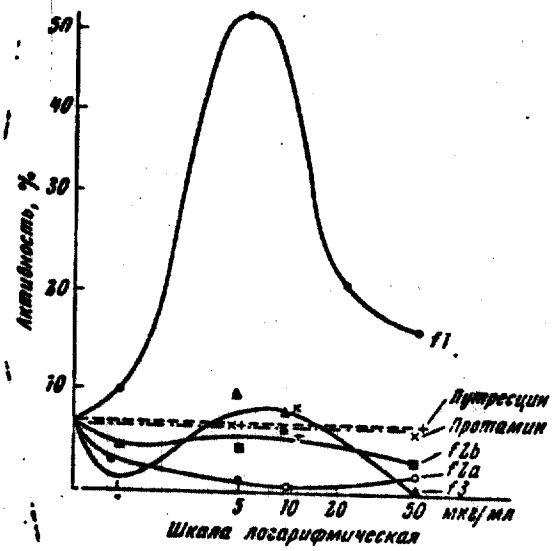
ды определения активности сперматозоидов по их подвижности подробно описаны ранее [10, 13]. Для хранения семя разводили равным объемом глюкозо-бикарбонат-борной среды (ГББ) [10, 13]. Часть опытов проводили с семенными клетками, отделенными от плазмы центрифугированием семени при 600 *g* в течение 10 *мин* с последующим суспендированием осадка в физиологическом растворе. Результаты обрабатывали статистически по схеме двухфакторного дисперсионного анализа.



Активность сперматозоидов в присутствии гистонов, протамина и путресцина, после 48 часов хранения цельного семени в среде ГББ при 18—22°

Данные, представленные на рисунке, свидетельствуют о том, что только фракция лизинбогатого гистона *f*1 в концентрации 5—25 *мкг/мл* обладает четко выраженным стабилизирующим действием на подвижность сперматозоидов. Весьма низкие значения оптимальной стабилизирующей концентрации гистона *f*1—5 *мкг/мл*, а также высокая степень очистки последнего, достигаемая при получении по методу Джонса, позволяют полагать, что наблюдаемый эффект не связан с примесями негистонной природы. Ни другие фракции гистонов тимуса (*f*2*a*, *f*2*b* и *f*3), ни протамины, ни диамин — путресцин не обладают таким действием. Степень стабилизации не зависит от того, добавляется ли гистон *f*1 в цельное семя или в суспензию спермиев в физиологическом растворе. Фракция *f*2*a* во всех испытанных концентрациях и 50 *мкг/мл* гистона *f*3 подавляют активность спермиев. Фракция *f*2*b*, протамин и путресцин не оказывают заметного действия.

Столь выраженная специфичность действия гистона *f*1, пожалуй, не имеет прецедентов. Во всех известных нам по данным литературы системах имеет место лишь количественное различие в действии разных фракций. В данном же случае наблюдается даже противоположная направленность действия *f*1 по сравнению с другими фракциями в широком диапазоне концентраций.

Пока нет данных для суждений о механизме стабилизирующего действия фракции *f*1, а тем более причинах специфичности этого эффекта. Правомочны предположения и о стабилизирующем действии на третичную структуру ДНК спермиев, и о действии гистонов на состояние его клеточных мембран, и многие другие.

Мы попытались, далее, сделать первые шаги в изучении основных стадий стабилизирующего действия. При изучении действия гистонов на бактерии и вирусы выявлены стадии обратимого и необратимого эффекта гистонов [14, 15]. Это удалось сделать, используя, в частности, метафосфат натрия, который образует с гистоном при pH 6—7,5 прочные нерастворимые комплексы и в то же время нейтрален в этих условиях по отношению к испытывавшимся микроорганизмам [12, 14, 15].

Результаты опытов, представленные в табл. 1, показывают, что добавление метафосфата натрия одновременно с гистонами *f*1 или через 15—30 *мин* значительно снижает стабилизирующий эффект последнего. Однако добавление метафосфата натрия через 45 *мин* и позже уже не

Таблица 1

Влияние метафосфата натрия на стабилизацию спермиев гистоном f_1

Концентрация, *мкг/мл*		Интервал между введением гистона и метафосфата, *мин*	Доля подвижных спермиев через 3 часа инкубации, %	Концентрация, *мкг/мл*		Интервал между введением гистона и метафосфата, *мин*	Доля подвижных спермиев через 3 часа инкубации, %
гистон f_1	метафосфат натрия			гистон f_1	метафосфат натрия		
5	125	0	8	5	125	90	51
5	125	15	7	5	0	—	66
5	125	30	7	0	125	—	10
5	125	45	57	0	0	—	8
5	125	60	56				

может повлиять на стабилизирующее действие гистона. Обращает на себя внимание резкий переход от стадии полной обратимости к стадии почти полной необратимости, наблюдаемый через 30—45 *мин* взаимодействия. Очевидно, к этому времени гистоны прочно связываются с какими-то компонентами клетки и метафосфат уже не способен «вырвать» гистон из образовавшегося комплекса или не может проникнуть в ту область клетки, где образуется этот комплекс.

Необходимо было далее сопоставить обращающее действие метафосфата с эффектом простого удаления гистона из среды на разных сроках после начала хранения. Удаление большей части гистона достигалось заменой среды после центрифугирования. Чтобы избежать повторного центрифугирования, травмирующего сперматозоиды, эти опыты ставили не с суспензией спермиев, а с цельным семенем, разбавленным раствором гистона. Через определенные интервалы времени пробы центрифугировали и полученные осадки суспендировали в физиологическом растворе без гистона. Такой же обработке подвергались и контрольные пробы. После этого в среде не могло содержаться более 0,5 *мкг/мл* гистона $f1$, что заведомо недостаточно для стабилизации. Отсчет времени вели от начала разбавления цельного семени. Как показано в табл. 2, удаление большей части гистона даже через 5 *мин* контакта уже не могло снять стабилизирующего действия. Очевидно, гистоны быстро сорбируются клеткой. Это соответствует данным Гирша [16] и других авторов изучавших динамику воздействия гистонов с клетками бактерий. Если сопоставить эти данные с описанными выше результатами опытов с метафосфатом, то можно сделать вывод, что действие последнего нельзя свести к связыванию свободного гистона среды. В противном случае его эффект был бы таким же, как в опытах по замене среды. Очевидно, метафосфат натрия способен взаимодействовать с гистонами, уже связавшимися с клеткой. Однако гистоны, проникая в клетку, не сразу образуют комплексы, устойчивые к метафосфату натрия, и в течение 30 *мин* еще доступны действию последнего.

Таблица 2

Влияние удаления гистона f_1 из среды на стабильность спермиев

Исходная концентрация гистона 5 *мкг/мл*

Время замены исходной среды на среду без гистона, *мин*	Доля подвижных спермиев через 6 часов инкубации, %
0	2
5	13
10	10
15	16
45	20
60	13
90	20
Не заменялась	16

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SPECIFICITY AND REVERSIBILITY OF STABILIZATION OF SPERM CELLS BY CERTAIN HISTONS

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Lysine-rich histone (f_1) from calf thymus stabilizes sperm cells of boar, according to mobility test. The effect is specific: all other histones of thymus (f_{2a}, f_{2b}, f_3) protamine and putrescine do not exert such action. In contrast, arginine-reach histones f_{2a} and f_3 suppress the activity of sperm cells. Effect of histone f_1 bound to sperm cells is completely relieved by sodium metaphosphate during the first 30 *min* After 45 *min* exposition the action cannot be reversed.

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Möglichkeiten des Polyphosphateinsatzes in der Tierernährung

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2. Mitteilung

Untersuchungen über die ^{32}P-Aufnahme aus markiertem Ortho-, Pyro- und Graham'schen Polyphosphat nach oraler Infusion an nüchterne Ratten

1. Einleitung

In der 1. Mitteilung zu diesem Themenkreis wurde über Bilanzversuche mit verschiedenen Polyphosphaten an Ratten berichtet [4]. Um die Ausnutzbarkeit des Polyphosphat-Phosphors durch das monogastrische Tier weiter zu untersuchen, wurde mit ^{32}P-markiertem Ortho-, Pyro- und Graham'schem Polyphosphat der Durchlauf des Phosphors dieser Verbindungen durch den Verdauungstrakt und die P-Einlagerung in verschiedene Organe bzw. Körperteile von Ratten in bestimmten Zeitabständen studiert. Dabei wurden zunächst die genannten Phosphate den Tieren in wäßriger Lösung nach einer 16stündigen Hungerperiode mittels einer Sonde eingegeben. Über die Verabreichung derselben in fester Form über das Futter wird in einer weiteren Mitteilung berichtet.

2. Material und Methodik

Als Versuchstiere dienten wie in den Bilanzversuchen 4–6 Wochen alte Ratten, die unserer institutseigenen Zucht entstammten. Bis zu Beginn des Versuchs erhielten die Tiere die genannte Zuchtdiät (siehe 1. Mitteilung); Wasser wurde ad libitum verabreicht. Insgesamt standen 44 Tiere zur Verfügung, die in drei Gruppen zu 14–16 Tieren aufgeteilt wurden.

Die Haltung der Tiere bis zur Applikation der Phosphatlösungen erfolgte in Gruppen zu je 2 Tieren in Stoffwechselkäfigen. Einen Tag vor der Eingabe der Phosphatlösungen wurde den Tieren das Futter entzogen und 16 Stunden danach

Phos

erhielten die Tiere der Gruppe I (14 Tiere) je 100 g Körpermasse 1 ml Orthophosphatlösung ($Na_2H^{32}PO_4$), die der Gruppe II (16 Tiere) je 100 g Körpermasse 1 ml Pyrophosphatlösung ($Na_4{}^{32}P_2O_7$) und die der Gruppe III (14 Tiere) je 100 g Körpermasse 1 ml Grahamsalzlösung [$(Na^{32}PO_3)_n \cdot H_2O$] oral infundiert.

Die spezifische Aktivität und der Phosphorgehalt der einzelnen Lösungen sind in Tabelle 1 dargestellt.

Tabelle 1

Phosphorgehalt und spezifische Aktivität der infundierten Lösungen

Phosphat	P-Gehalt mg/ml	spez. Aktivität Imp./Min. je mg P
$Na_2H^{32}PO_4$	11	53727
$Na_4{}^{32}P_2O_7$	16.5	23636
$(Na^{32}PO_3)_n \cdot H_2O$	17.7	31[illegible]7

Jeweils 2 bzw. 4 Tiere einer Gruppe wurden 15, 30, 60, 120, 240 und 360 Minuten nach der Applikation getötet, entblutet und Impulsratenmessungen in folgenden Organen bzw. Körperbestandteilen durchgeführt:

Blut, Leber, Niere, linker Tibia-Knochen, Muskel sowie Inhalt und Wand des gesamten Verdauungstraktes. Eine Weiterverfolgung über einen längeren Zeitraum hinweg wurde nicht durchgeführt, da dann mit einer Rückausscheidung von markiertem Phosphor in den Verdauungskanal zu rechnen ist, wodurch die entsprechenden Werte für den Inhalt der verschiedenen Darmabschnitte verfälscht würden. Die in 6 Stunden nach der Eingabe erfolgende endogene Rückexkretion von ^{32}P in den Verdauungskanal beträgt dagegen nur etwa 1.5% der applizierten Dosis (CRAMER [2]).

Der Verdauungstrakt wurde dabei in folgende Abschnitte geteilt: Magen, Dünndarmabschnitte I, II und III (der Dünndarm wurde in drei gleiche Teile zerlegt, die als aufeinanderfolgende Abschnitte I, II und III bezeichnet werden) und Dickdarm, wobei sowohl die Aktivität der Wände der entsprechenden Abschnitte als auch die der Inhalte derselben bestimmt wurde.

Blut (nach Zusatz von Natriumcitrat zur Verhinderung der Gerinnung), Harn und die Inhalte der genannten Abschnitte des Verdauungstrakts (als wäßrige Suspension) wurden sofort gemessen. Die Knochenproben wurden trocken verascht, mit HCl aufgenommen und anschließend gemessen. Die übrigen Organe bzw. Organteile wurden mit einem Gemisch von H_2SO_4 + HNO_3 hydrolisiert und die Hydrolysate gemessen. Die Impulsratenmessungen erfolgten nach der von BERGNER und HOCK [1] beschriebenen Methodik.

3. Ergebnisse

3.1. Verdauungstrakt

In Tabelle 2 und Abb. 1 und 2 sind die Ergebnisse der Impulsratenmessung 15 Minuten nach der Applikation dargestellt. Diese, wie auch alle anderen Werte geben die wiedergefundene Aktivität in Prozenten der applizierten Dosis an. Sie stellen Durchschnittswerte von jeweils 2 bzw. 4 Tieren dar. Die nach 15 Minuten im Inhalt des Verdauungstraktes wiedergefundene Aktivität machte bei den Orthophosphattieren 47,3%, bei den Pyrophosphattieren 32,8% und bei den Grahamsalztieren 29,3% aus. Im Gegensatz dazu wurden in den Wänden des Verdauungskanals in Gruppe I (Orthophosphat) insgesamt 25,4%, in Gruppe II (Pyrophosphat) 33,8% und in Gruppe III (Graham'sches Polyphosphat) 37,8% der applizierten Impulse wiedergefunden.

In Tabelle 3 und Abb. 3 und 4 sind die Ereignisse der Messungen 30 Minuten nach der Infusion dargestellt. Die im Inhalt der verschiedenen Teile des Verdauungskanals wiedergefundene Aktivität war in Gruppe I um fast 20% geringer (29,8 gegenüber 47,3%) als 15 Minuten nach der Eingabe der Phosphatlösung. In Gruppe II war dagegen eine leichte (38,6% gegenüber 32,8%) und in Gruppe III eine starke Zunahme (41,0% gegenüber 29,3%) im Vergleich zum ersten Meßzeitpunkt festzustellen. Die Unterschiede in den Werten der Wände des Verdauungstrakts waren dagegen geringer als 15 Minuten nach der Applikation (31,5% in Gruppe I — 35,6% in Gruppe III), wobei der höhere Prozentsatz in Gruppe III gegenüber den anderen Gruppen besonders durch die sehr hohen Werte in den letzten Abschnitten des Dünndarms bedingt ist.

Nach einer Stunde waren in Gruppe I 40,0%, in Gruppe II 36,7% und in Gruppe III 35,6% der eingegebenen Dosis im Inhalt des Verdauungskanals wiederzufinden. In der Wand desselben wurden zu diesem Zeitpunkt kaum Unterschiede zwischen den einzelnen Gruppen festgestellt. Die Einzelergebnisse sind in Tabelle 4 und Abb. 5 und 6 dargestellt.

Nach 2 Stunden (Tabelle 5 und Abb. 7 und 8) wurden in Gruppe I 30,9%, in Gruppe II 31,3% und in Gruppe III 39,3% der applizierten Impulse im Inhalt des Verdauungskanals wiedergefunden. Der Hauptanteil derselben wurde dabei im Dickdarminhalt gefunden, wobei besonders die Tiere der Gruppe III, wie es sich schon am vorherigen Meßzeitpunkt (1 Stunde nach Infusion) andeutete, wesentlich höhere Werte als die der beiden anderen Gruppen zeigten. In den Wänden des Verdauungskanals wurden 2 Stunden nach der Eingabe der Phosphatlösungen bei den Tieren der Gruppe I 17,5%, der Gruppe II 18,9% und der Gruppe III 22,4% der eingegebenen Dosis wiedergefunden. Während die entsprechenden Werte bis 1 Stunde nach der Eingabe relativ konstant blieben, zeigte sich nach 2 Stunden ein sehr starker Abfall derselben.

Nach 4 Stunden (Tabelle 6 und Abb. 9 und 10) wurden im Inhalt des Verdauungstraktes bei den Tieren der Gruppe I noch 23,9% und bei denen der Gruppe II noch 26,2% der applizierten Dosis wiedergefunden. Gegenüber dem

Tabelle 2

Wiedergefundene Aktivität (in % der applizierten Dosis) im Inhalt und in der Wand des Verdauungstrakts 15 Minuten nach Infusion der Phosphatlösungen

Gruppe Nr.	Ratte Nr.	infundierte P-Verbindung	Ø Körpermasse der Tiere (g)	wiedergefundene Aktivität (in % der appl. Dosis) im Inhalt des Magens	Darmab. I	Darmab. II	Darmab. III	Dickdarms	gesamten Verdauungstrakts
I	1–4	$Na_2H^{32}PO_4$	52,6	34,8	4,2	5,5	1,0	0,0	47,3
II	5–6	$Na_4{}^{32}P_2O_7$	40,7	22,7	2,3	2,5	4,0	1,3	32,8
III	7–10	$(Na^{32}PO_3)_n \cdot H_2O$	67,1	17,3	1,6	0,0	2,4	1,2	26,4

				wiedergefundene Aktivität (in % der appl. Dosis) in der Wand des Magens	Darmab. I	Darmab. II	Darmab. III	Dickdarms	gesamten Verdauungstrakts
I	1–4	wie oben	wie oben	7,9	5,3	6,0	4,7	1,8	25,7
II	5–6	„ „	„ „	8,3	6,7	6,7	10,0	1,2	33,8
III	7–10	„ „	„ „	6,0	3,4	13,5	14,4	0,5	37,8

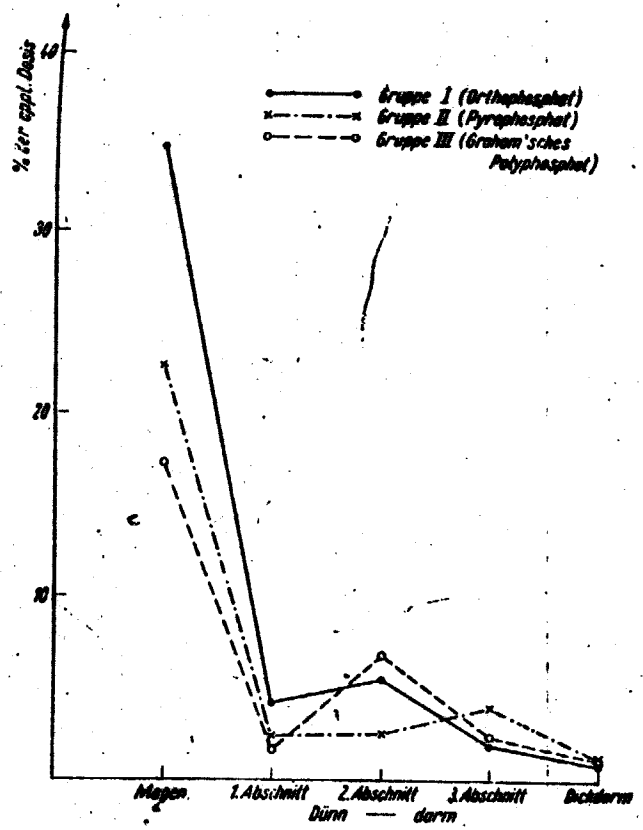

Abb. 1. Wiedergefundene Aktivität (in % der appl. Dosis) im Inhalt der verschiedenen Abschnitte des Verdauungstrakts 15 Min. nach Infusion der Phosphatlösungen

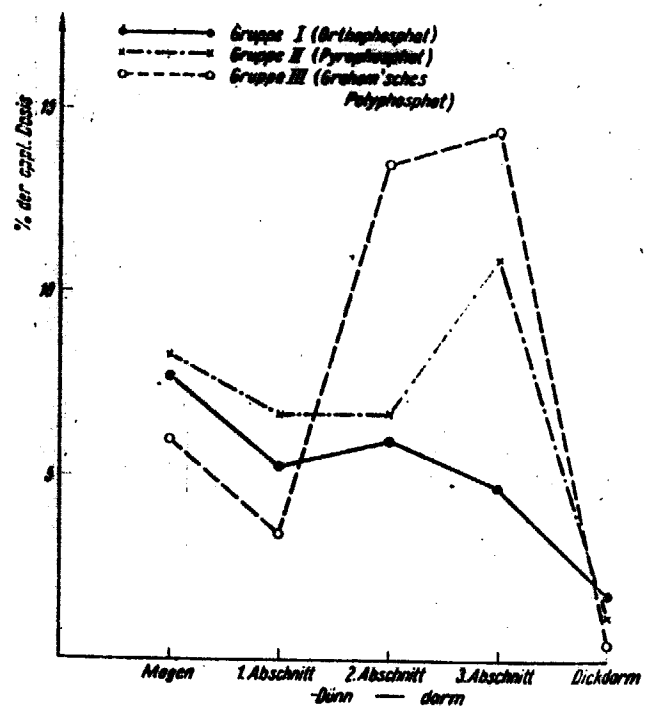

Abb. 2. Wiedergefundene Aktivität (in % der appl. Dosis) in der Wand der verschiedenen Abschnitte des Verdauungstrakts 15 Min. nach Infusion der Phosphatlösungen

Tabelle 3

Wiedergefundene Aktivität (in % der applizierten Dosis) im Inhalt und in der Wand des Verdauungstrakts 30 Minuten nach Infusion der Phosphatlösungen

Gruppe Nr.	Ratte Nr.	infundierte P-Verbindung	Ø Körpermasse der Tiere (g)	wiedergefundene Aktivität (in % der appl. Dosis) im Inhalt des Magens	Darmab. I	Darmab. II	Darmab. III	Dickdarms	gesamten Verdauungstrakts
I	11–12	$Na_2H^{32}PO_4$	54,5	19,6	2,1	3,9	3,9	0,4	29,9
II	13–16	$Na_4{}^{32}P_2O_7$	64,7	18,6	4,9	8,0	6,0	1,1	38,6
III	17–18	$(Na^{32}PO_3)_n \cdot H_2O$	40,8	26,9	0,8	1,8	11,1	0,3	40,9

				wiedergefundene Aktivität (in % der appl. Dosis) in der Wand des Magens	Darmab. I	Darmab. II	Darmab. III	Dickdarms	gesamten Verdauungstrakts
I	11–12	wie oben	wie oben	11,4	5,2	5,8	7,5	1,6	31,5
II	13–16	„ „	„ „	7,9	7,5	7,6	9,4	1,3	33,7
III	17–18	„ „	„ „	8,1	3,9	6,0	16,9	0,6	35,5

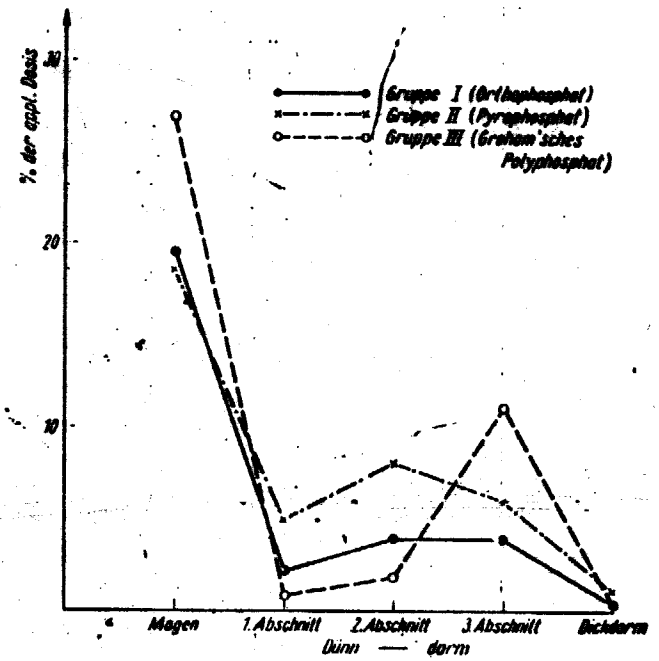

Abb. 3. Wiedergefundene Aktivität (in % der appl. Dosis) im Inhalt der verschiedenen Abschnitte des Verdauungstrakts 30 Min. nach Infusion der Phosphatlösungen

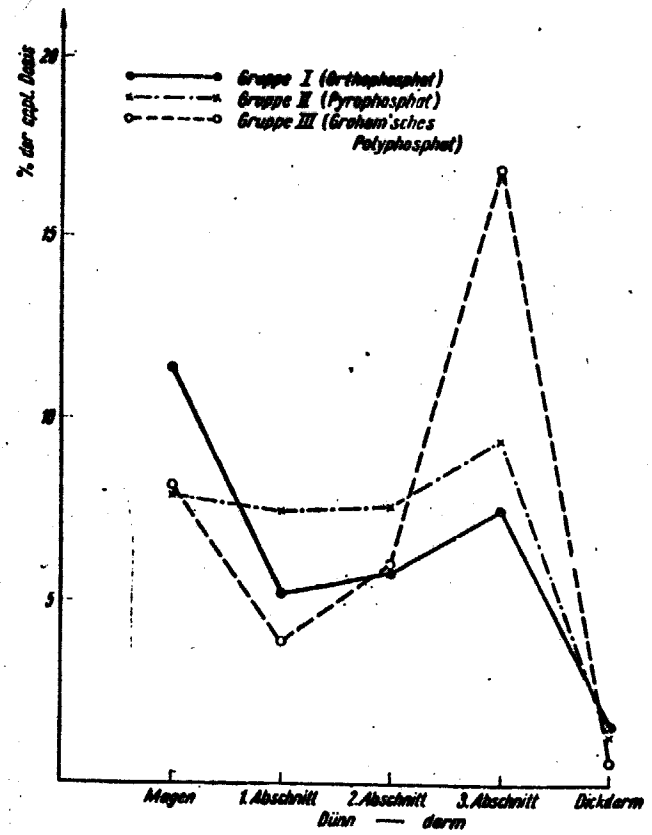

Abb. 4. Wiedergefundene Aktivität (in % der appl. Dosis) in der Wand der verschiedenen Abschnitte des Verdauungstrakts 30 Min. nach Infusion der Phosphatlösungen

Tabelle 4

Wiedergefundene Aktivität (in % der applizierten Dosis) im Inhalt und in der Wand des Verdauungstrakts
1 Stunde nach Infusion der Phosphatlösungen

Gruppe Nr.	Ratte Nr.	infundierte P-Verbindung	Ø Körpermasse der Tiere (g)	wiedergefundene Aktivität (in % der appl. Dosis) im Inhalt des Magens	Darmab. I	Darmab. II	Darmab. III	Dickdarms	gesamten Verdauungstrakts
I	19–20	$Na_2H^{32}PO_4$	44,5	20,7	1,1	3,7	5,9	8,0	39,4
II	21–24	$Na_4{}^{32}P_2O_7$	67,2	6,7	5,4	4,9	8,0	11,7	36,7
III	25–26	$(Na^{32}PO_3)_n . H_2O$	40,5	7,3	0,5	1,7	3,5	23,4	35,4

				wiedergefundene Aktivität (in % der appl. Dosis) in der Wand des Magens	Darmab. I	Darmab. II	Darmab. III	Dickdarms	gesamten Verdauungstrakts
I	19–20	wie oben	wie oben	8,0	4,0	5,7	11,2	4,9	33,8
II	21–24	„ „	„ „	4,4	9,4	6,3	7,9	2,9	30,9
III	25–26	„ „	„ „	4,2	2,6	5,7	7,8	13,1	33,4

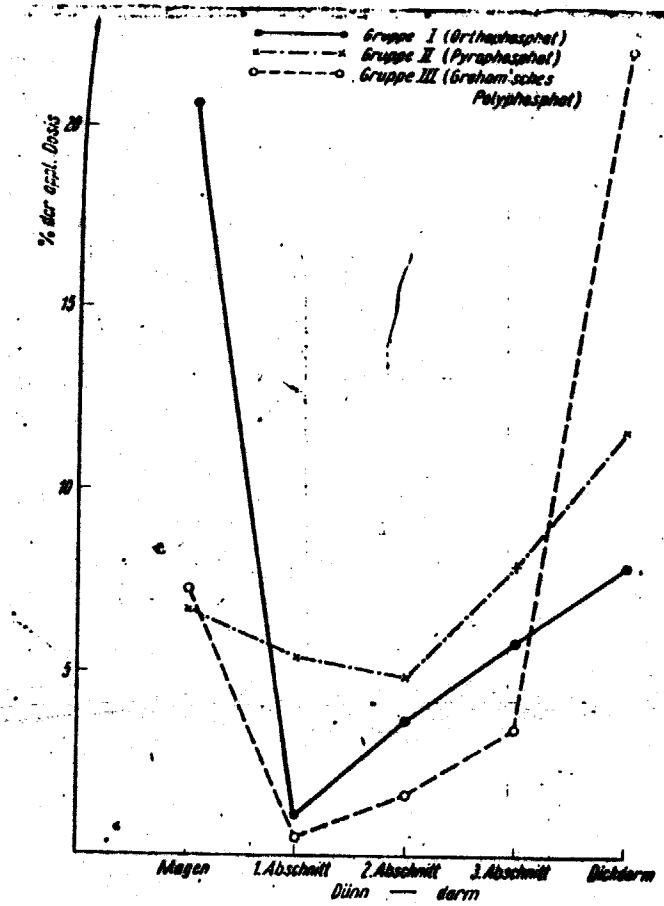

Abb. 5. Wiedergefundene Aktivität (in % der appl. Dosis) im Inhalt der verschiedenen Abschnitte des Verdauungstrakts 1 Std. nach Infusion der Phosphatlösungen

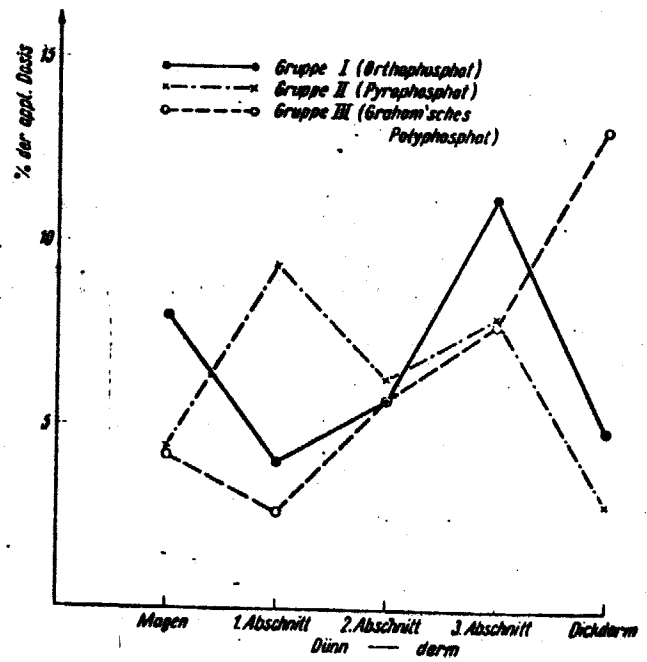

Abb. 6. Wiedergefundene Aktivität (in % der appl. Dosis) in der Wand der verschiedenen Abschnitte des Verdauungstrakts 1 Std. nach Infusion der Phosphatlösungen

Tabelle 5

Wiedergefundene Aktivität (in % der applizierten Dosis) im Inhalt und in der Wand des Verdauungstrakts 2 Stunden nach Infusion der Phosphatlösungen

Gruppe Nr.	Ratte Nr.	infundierte P-Verbindung	Ø Körpermasse der Tiere (g)	wiedergefundene Aktivität (in % der appl. Dosis im Inhalt des Magens	Darmab. I	Darmab. II	Darmab. III	Dickdarms	gesamten Verdauungstrakts
I	27–28	$Na_2H^{32}PO_4$	57,7	1,3	0,8	3,1	3,5	22,1	30,8
II	29–30	$Na_4{}^{32}P_2O_7$	61,7	0,4	0,4	1,5	6,3	22,7	31,3
III	31–32	$(Na^{32}PO_3)_n \cdot H_2O$	55,2	2,1	0,6	1,3	2,9	32,5	39,4

				wiedergefundene Aktivität (in % der appl. Dosis) in der Wand des Magens	Darmab. I	Darmab. II	Darmab. III	Dickdarms	gesamten Verdauungstrakts
I	27–28	wie oben	wie oben	1,2	2,9	2,8	6,3	4,2	17,4
II	29–30	„ „	„ „	1,1	3,2	2,5	6,4	5,7	18,9
III	31–32	„ „	„ „	2,2	2,0	2,6	5,8	9,7	22,3

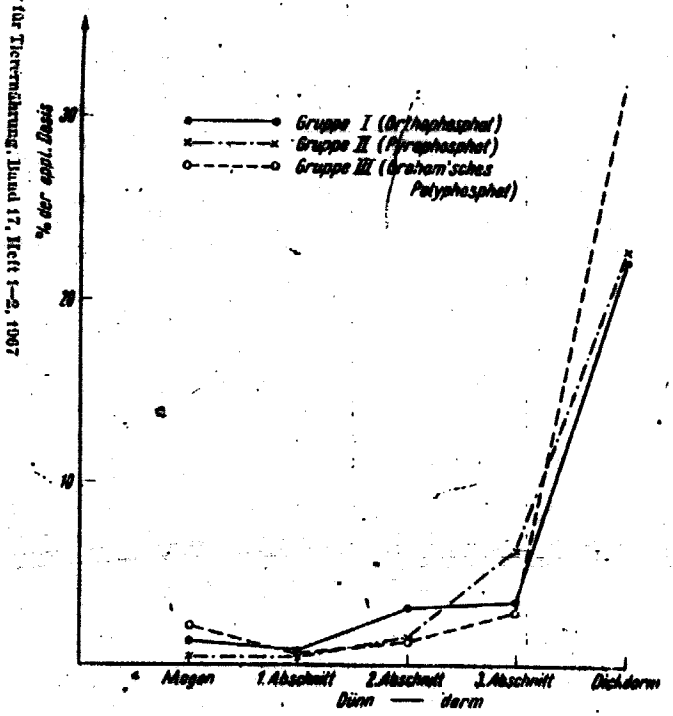

Abb. 7. Wiedergefundene Aktivität (in % der appl. Dosis) im Inhalt der verschiedenen Abschnitte des Verdauungstrakts 2 Std. nach Infusion der Phosphatlösungen

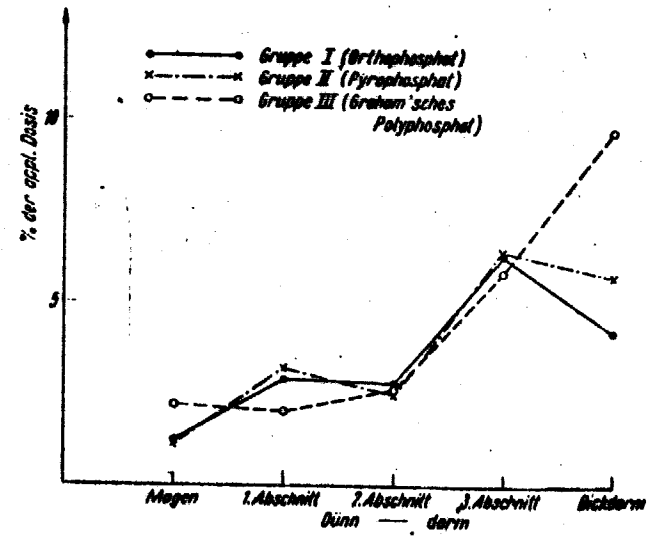

Abb. 8. Wiedergefundene Aktivität (in % der appl. Dosis) in der Wand der verschiedenen Abschnitte des Verdauungstrakts 2 Std. nach Infusion der Phosphatlösungen

Tabelle 6

Wiedergefundene Aktivität (in % der applizierten Dosis) im Inhalt und in der Wand des Verdauungstrakts 4 Stunden nach Infusion der Phosphatlösungen

Gruppe Nr.	Ratte Nr.	infundierte P-Verbindung	Ø Körpermasse der Tiere (g)	wiedergefundene Aktivität (in % der appl. Dosis) im Inhalt des Magens	Darmab. I	Darmab. II	Darmab. III	Dickdarms	gesamten Verdauungstrakts
I	33–34	$Na_2H^{32}PO_4$	51,0	0,1	0,3	0,3	1,8	21,4	23,9
II	35–36	$Na_4{}^{32}P_2O_7$	57,0	0,1	0,3	0,5	1,9	23,4	26,2
III	37–38	$(Na^{32}PO_3)_n \cdot H_2O$	61,2	0,3	0,2	0,5	3,7	33,5	38,2

				wiedergefundene Aktivität (in % der appl. Dosis) in der Wand des Magens	Darmab. I	Darmab. II	Darmab. III	Dickdarms	gesamten Verdauungstrakts
I	33–34	wie oben	wie oben	0,8	2,4	1,8	1,3	6,2	12,5
II	35–36	„ „	„ „	0,9	2,4	1,7	1,4	4,3	10,7
III	37–38	„ „	„ „	0,4	0,8	0,6	1,9	11,5	15,2

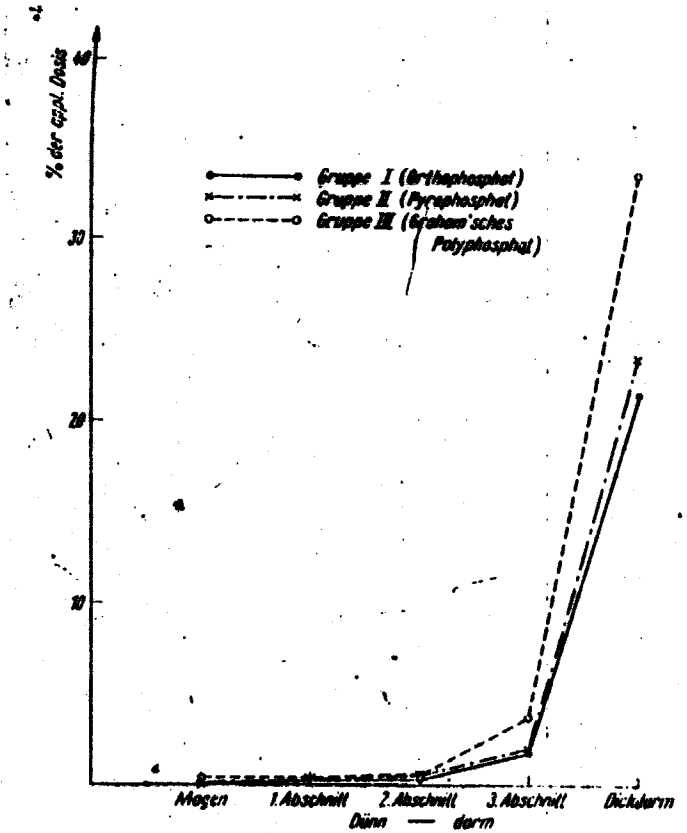

Abb. 9. Wiedergefundene Aktivität (in % der appl. Dosis) im Inhalt der verschiedenen Abschnitte des Verdauungstrakts 4 Std. nach Infusion der Phosphatlösungen

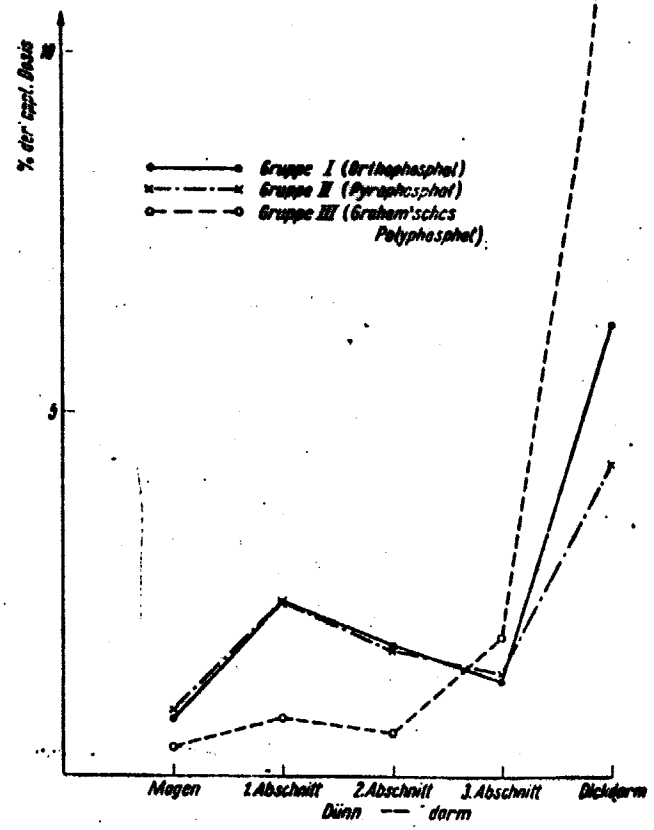

Abb. 10. Wiedergefundene Aktivität (in % der appl. Dosis) in der Wand der verschiedenen Abschnitte des Verdauungstrakts 4 Std. nach Infusion der Phosphatlösungen

vorherigen Meßzeitpunkt zeigten sich in beiden Gruppen etwa gleiche Abnahmen. Dagegen blieb die wiedergefundene Gesamtaktivität des Verdauungstraktinhalts bei den Tieren der Gruppe III ziemlich auf gleicher Höhe (38.2% gegenüber 39.3%). Die in den Wänden des Verdauungskanals nach 4 Stunden wiedergefundene Aktivität betrug 12.6%, 10.8% und 15.3% der eingegebenen Dosis für die Tiere der Gruppen I–III. zeigte also weiterhin eine abnehmende Tendenz.

6 Stunden nach der Infusion (Tabelle 7 und Abb. 11 und 12) waren bei den Tieren der Gruppe I noch 27,0% und bei denen der Gruppe II noch 26.2% der applizierten Mengen im Inhalt des Verdauungstrakts vorhanden. Bei den Tieren der Gruppe III lag die wiedergefundene Gesamtaktivität wesentlich höher – der Wert lag bei 47,9%. Naturgemäß wurde mit fortschreitender Zeitdauer nach der Applikation der Phosphatlösungen der Hauptteil der wiedergefundenen Impulse/Minute in den hinteren Abschnitten des Verdauungstrakts festgestellt. In den Wänden des Verdauungskanals wurden bei den Tieren der Gruppe I noch 10,6%, bei denen der Gruppe II noch 12.0% und bei den Tieren der Gruppe III noch 8.7% der eingegebenen Aktivität wiedergefunden, also keine nennenswerten Unterschiede mehr festgestellt. Die in den verschiedenen Zeitabständen nach der Infusion der Phosphatlösung im Gesamtinhalt und der gesamten Wand des Verdauungstrakts wiedergefundenen Aktivitätswerte sind in Abb. 13 und 14 zusammenfassend dargestellt.

3.2. Organe bzw. Organteile

Die in den genannten Zeitabständen nach der Applikation in den verschiedenen Organen bzw. Organteilen wiedergefundenen prozentualen Anteile der eingegebenen Aktivität sind in den Tabellen 8–13 und den Abbildungen 15–19 dargestellt.

3.2.1. Blut (s. Abb. 15)

15 Minuten nach der Applikation wurden in Gruppe I 0,17%, in Gruppe II 0,37% und in Gruppe III 0,03% der eingegebenen Dosis/ml Blut wiedergefunden. Während bei den Tieren der Gruppe I das Maximum nach 2 Stunden mit 0.40% erreicht wurde und die Werte danach wieder etwas abfielen (bis auf 0,26% nach 6 Stunden), blieben die Werte bei den Tieren der Gruppe II ziemlich konstant. Der höchste Wert wurde schon nach 15 Minuten mit 0.37% gefunden, nach 6 Stunden wurden hier noch 0,28% der applizierten Dosis festgestellt. Die Werte in Gruppe III lagen in dem untersuchten Zeitraum mit 0,03% der eingegebenen Dosis nach 15 Minuten bis 0,045% nach 6 Stunden relativ konstant, sie lagen jedoch zu allen Meßzeitpunkten um ein Vielfaches unter denen der beiden anderen Gruppen.

Tabelle 7

Wiedergefundene Aktivität (in % der applizierten Dosis) im Inhalt und in der Wand des Verdauungstrakts 6 Stunden nach Infusion der Phosphatlösungen

Gruppe Nr.	Ratte Nr.	infundierte P-Verbindung	ø Körpermasse der Tiere (g)	wiedergefundene Aktivität (in % der appl. Dosis) im Inhalt des Magens	Darmab. I	Darmab. II	Darmab. III	Dickdarms	gesamten Verdauungstrakts
I	39–40	$Na_2H^{32}PO_4$	62,0	0,1	0,05	0,05	0,4	26,4	27,0
II	41–42	$Na_4{}^{32}P_2O_7$	61,5	0,1	0,3	1,1	0,3	24,4	26,2
III	43–44	$(Na^{32}PO_3)_n . H_2O$	60,5	0,1	0,0	0,1	3,1	44,6	47,9

				wiedergefundene Aktivität (in % der appl. Dosis) in der Wand des Magens	Darmab. I	Darmab. II	Darmab. III	Dickdarms	gesamten Verdauungstrakts
I	39–40	wie oben	wie oben	0,6	1,7	1,6	1,2	5,4	10,5
II	41–42	„ „	„ „	0,6	2,1	1,5	1,4	6,4	12,0
III	43–44	„ „	„ „	0,3	0,5	0,4	0,7	6,8	8,7

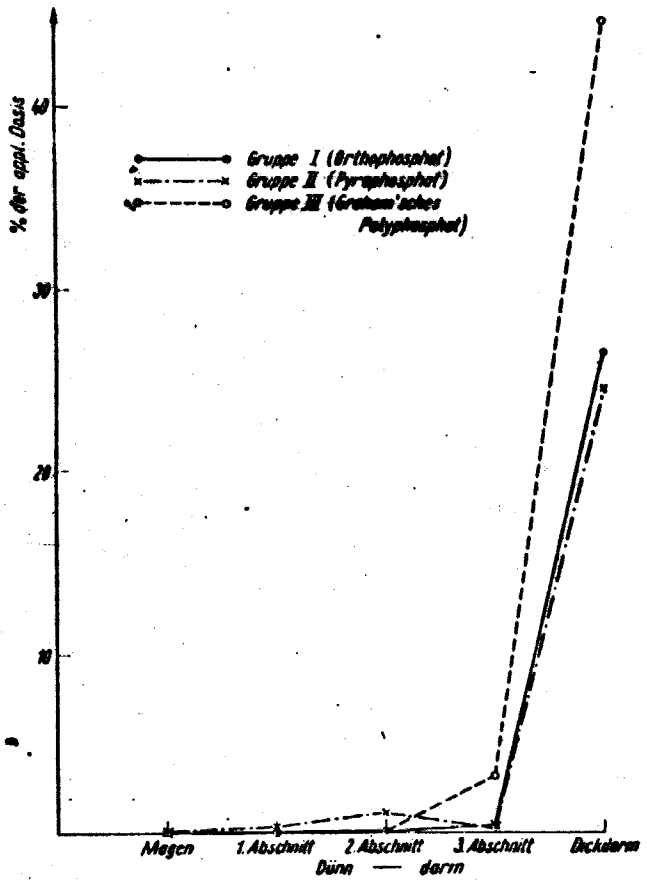

Abb. 11. Wiedergefundene Aktivität (in % der appl. Dosis) im Inhalt der verschiedenen Abschnitte des Verdauungstrakts

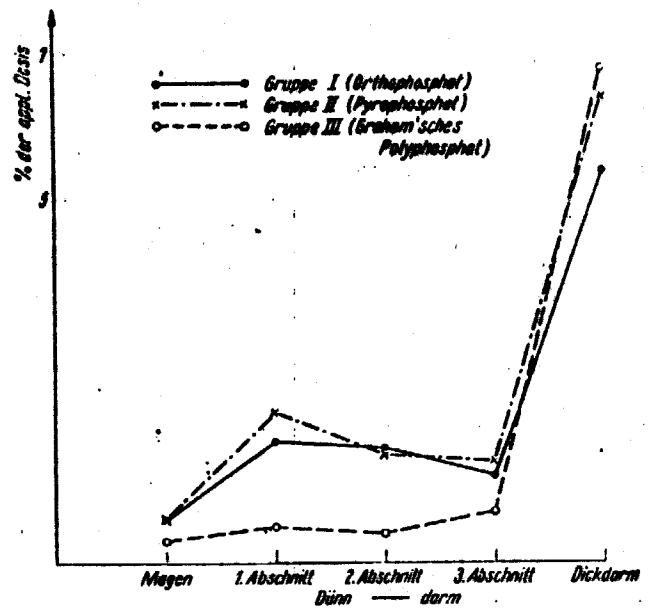

Abb. 12. Wiedergefundene Aktivität (in % der appl. Dosis) in der Wand der verschiedenen Abschnitte des Verdauungstraktes

Magen 1. Abschnitt 2. Abschnitt 3. Abschnitt Dickdarm
Dünn — darm

Abb. 11. Wiedergefundene Aktivität (in % der appl. Dosis) in Abhängigkeit der verschiedenen Abschnitte des Verdauungstraktes

Magen 1. Abschnitt
Dünn — darm

Abb. 12. Wiedergefundene Aktivität (in % der appl. Dosis) in Wand der verschiedenen Abschnitte des Verdauungstraktes

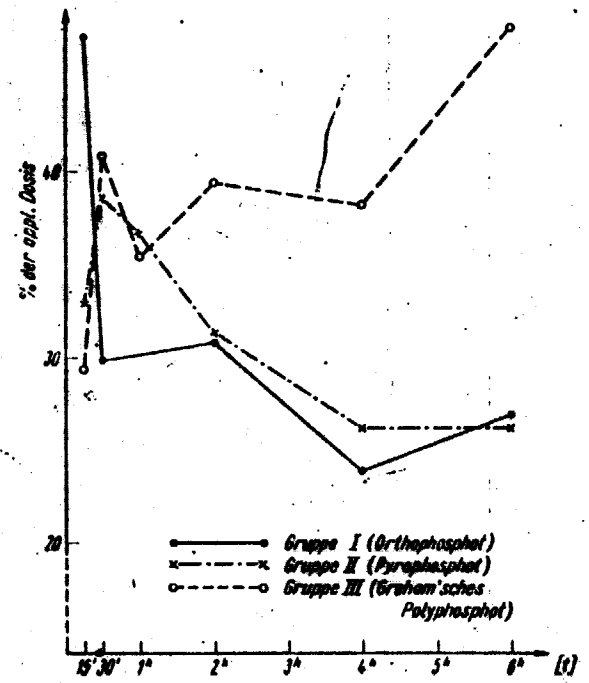

Abb. 13. Wiedergefundene Aktivität (in % der appl. Dosis) im Gesamtinhalt des Verdauungstrakts in den verschiedenen Zeitabständen nach der Infusion der Phosphatlösungen

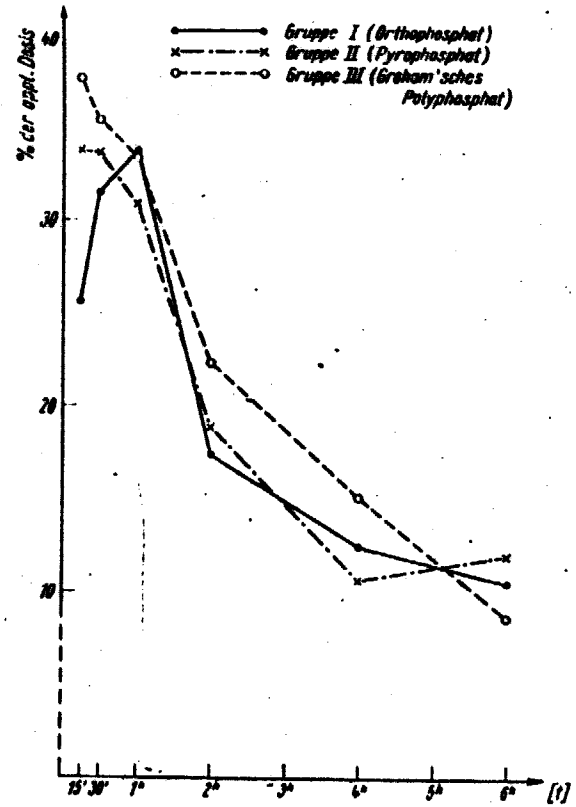

Abb. 14. Wiedergefundene Aktivität (in % der appl. Dosis) in der gesamten Wand des Verdauungstrakts in den verschiedenen Zeitabständen nach der Infusion der Phosphatlösungen

Tabelle 8

Wiedergefundene Aktivität (in % der applizierten Dosis) in Leber, Niere, Tibia, Blut (pro ml) und Muskel (pro g) 15 Minuten nach Eingabe der Phosphatlösungen

Gruppe Nr.	Ratte Nr.	wiedergefundene Aktivität (in % der appl. Dosis) in Leber	Niere	Tibia	Blut (pro ml)	Muskel (pro g)
I	1–4	0.85	1.31	0.20	0,17	0.04
II	5–6	1.51	0,60	0.11	0.37	0,04
III	7–10	0,79	0.55	0.015	0,03	0,005

Tabelle 9

Wiedergefundene Aktivität (in % der applizierten Dosis) in Leber, Niere, Tibia, Blut (pro ml) und Muskel (pro g) 30 Minuten nach Eingabe der Phosphatlösungen

Gruppe Nr.	Ratte Nr.	wiedergefundene Aktivität (in % der appl. Dosis) in Leber	Niere	Tibia	Blut (pro ml)	Muskel (pro g)
I	11–12	3.48	0,41	0.20	0.18	0.02
II	13–16	2.17	0.74	0.38	0.31	0.11
III	17–18	0.41	0,07	0.05	0.04	0,02

Tabelle 10

Wiedergefundene Aktivität (in % der applizierten Dosis) in Leber, Niere, Tibia, Blut (pro ml) und Muskel (pro g) 1 Stunde nach Eingabe der Phosphatlösungen

Gruppe Nr.	Ratte Nr.	wiedergefundene Aktivität (in % der appl. Dosis) in Leber	Niere	Tibia	Blut (pro ml)	Muskel (pro g
I	19–20	3.83	0,82	0,45	0,35	0,12
II	21–24	3,52	1.44	0,07	0.35	0.05
III	25–26	0,50	0.17	0,07	0,035	0.04

Tabelle 11

Wiedergefundene Aktivität (in % der applizierten Dosis) in Leber, Niere, Tibia, Blut (pro ml) und Muskel (pro g) 2 Stunden nach Eingabe der Phosphatlösungen

Gruppe Nr.	Ratte Nr.	wiedergefundene Aktivität (in % der appl. Dosis) in Leber	Niere	Tibia	Blut (pro ml)	Muskel (pro g)
I	27–28	4,28	1,20	0.80	0,40	0,09
II	29–30	3,57	0.87	0.82	0.30	0,05
III	31–32	0,30	0,31	0,16	0,035	0,03[2]

[2] Wert von 1 Tier

Tabelle 12

Wiedergefundene Aktivität (in % der applizierten Dosis) in Leber, Niere, Tibia, Blut (pro ml) und Muskel (pro g) 4 Stunden nach Eingabe der Phosphatlösungen

Gruppe Nr.	Ratte Nr.	wiedergefundene Aktivität (in % der appl. Dosis) in Leber	Niere	Tibia	Blut (pro ml)	Muskel (pro g)
I	33–34	3,86	0,88	1,23	0,35	0,07
II	35–36	4,05	0,76	1,13	0,27	0,035
III	37–38	0,68	0,26	0,16	0,045	0,045

Tabelle 13

Wiedergefundene Aktivität (in % der applizierten Dosis) in Leber, Niere, Tibia, Blut (pro ml) und Muskel (pro g) 6 Stunden nach Eingabe der Phosphatlösungen

Gruppe Nr.	Ratte Nr.	wiedergefundene Aktivität (in % der appl. Dosis) in Leber	Niere	Tibia	Blut (pro ml)	Muskel (pro g)
I	39–40	3,61	0,70	1,28	0,26	0,06
II	41–42	3,60	0,75	1,00	0,28	0,09
III	43–44	0,73	0,24	0,17	0,045	0,02

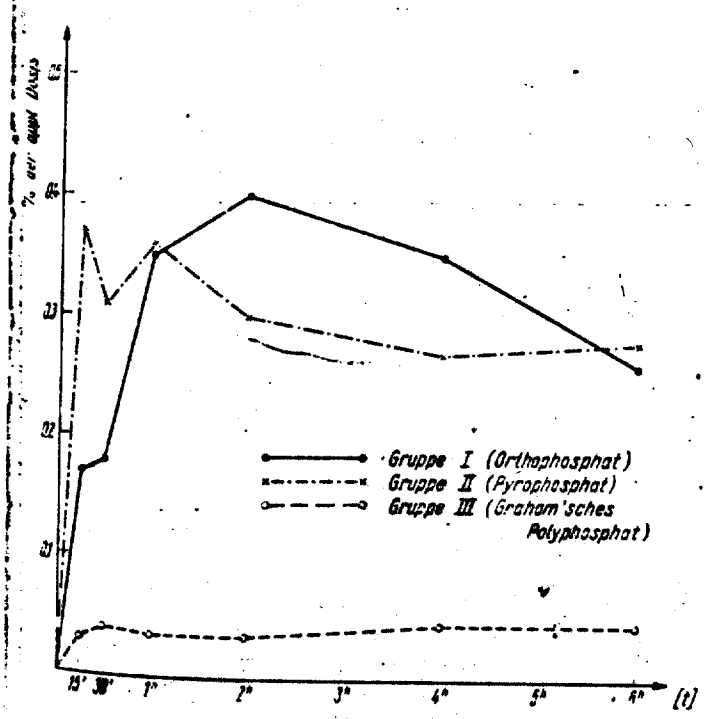

Abb. 15.
Wiedergefundene Aktivität (in % der appl. Dosis) in 1 ml Blut in den verschiedenen Zeitabständen nach der Infusion der Phosphatlösungen

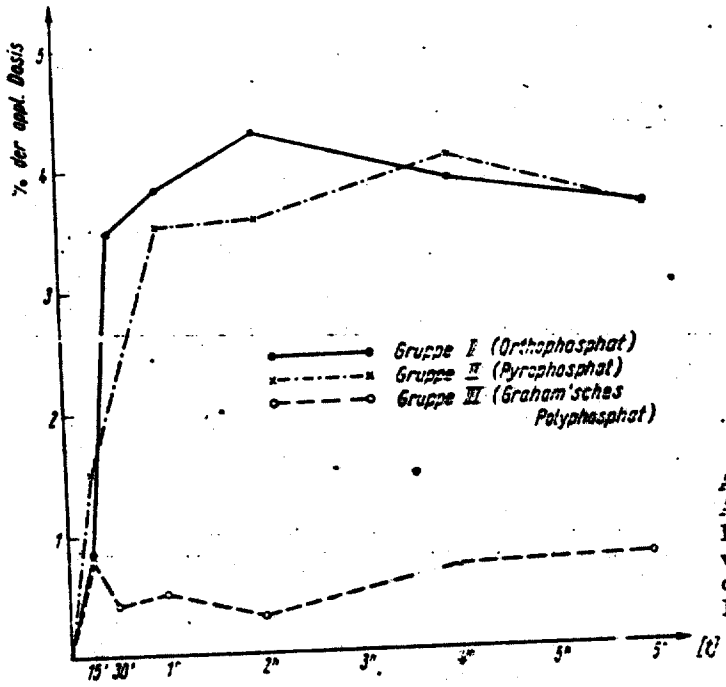

Abb. 16. Wiedergefunden Aktivität (in % der app. Dosis) in der Leber in den verschiedenen Zeitabständen nach der Infusion der Phosphatlösungen

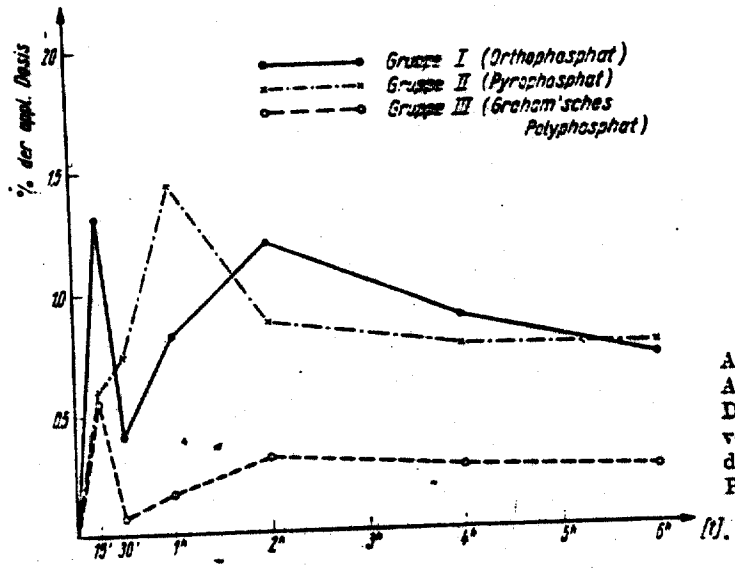

Abb. 17. Wiedergefundene Aktivität (in % der app. Dosis) in der Niere in den verschiedenen Zeitabständen nach der Infusion der Phosphatlösungen

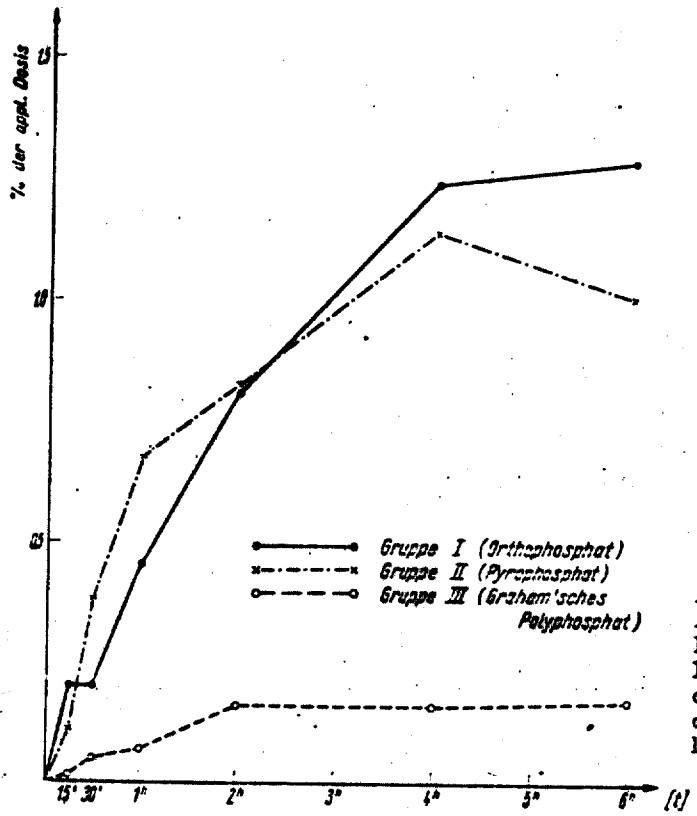

Abb. 18. Wiedergefundene Aktivität (in % der appl. Dosis) im linken Tibia-Knochen in den verschiedenen Zeitabständen nach der Infusion der Phosphatlösungen

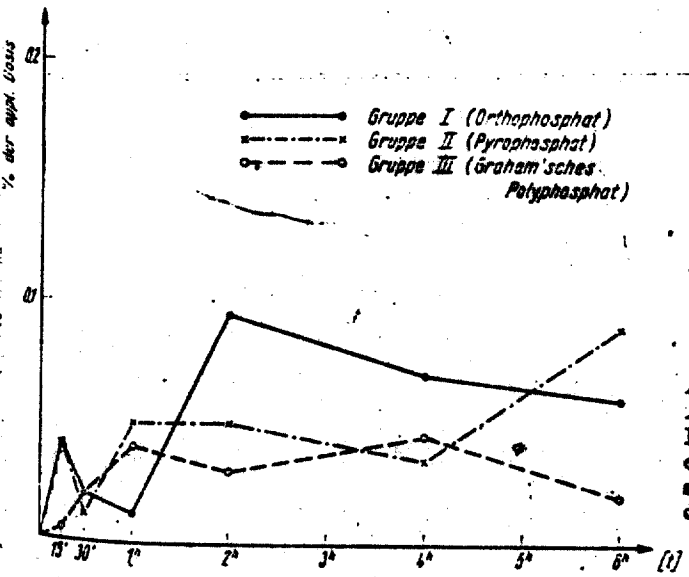

Abb. 19. Wiedergefundene Aktivität (in % der appl. Dosis) in 1 g Muskel in den verschiedenen Zeitabständen nach der Infusion der Phosphatlösungen

3.2.2. Leber (s. Abb. 16)

In der Leber wurden nach 15 Minuten bei den Tieren der Gruppe I 0.85%, bei denen der Gruppe II 1.51% und bei denen der Gruppe III 0.79% der eingegebenen Dosis wiedergefunden. Nach 30 Minuten stiegen die Werte bei den Tieren der Gruppe I auf 3.48% und bei denen der Gruppe II auf 2.17% an, während sie bei den Tieren der Gruppe III auf 0.41% absanken. 2 Stunden nach der Applikation war bei den Tieren der Gruppe I mit 4.28% das Maximum erreicht, während dasselbe bei denen der Gruppe II mit 4.05% 2 Stunden später zu verzeichnen war. Danach fielen die Werte in beiden Gruppen leicht ab und waren nach 6 Stunden mit 3,61% bzw. 3.60% praktisch gleich. Die Werte in Gruppe III erreichten nach 2 Stunden ein Minimum (0.30%), danach stiegen sie wieder auf 0.68% nach 4 Stunden bzw. 0.73% nach 6 Stunden an. Bis auf den ersten Meßzeitpunkt blieben die Werte jedoch ebenfalls um ein Vielfaches unter den entsprechenden Werten, die bei den Tieren der Gruppen I und II gefunden wurden.

3.2.3. Niere (s. Abb. 17)

Der 15 Minuten nach der Eingabe in der Niere wiedergefundene Anteil lag bei den Tieren der Gruppe I mit 1.21% mehr als doppelt so hoch als bei den Tieren der Gruppen II (0,60%) und III (0.55%). In Gruppe I fielen die Werte 30 Minuten nach der Eingabe auf 0.41% ab, stiegen dann bis 2 Stunden nach der Eingabe auf 1,20% an und nahmen dann wieder langsam ab. Nach 6 Stunden waren in der Niere 0.70% der applizierten Dosis wiederzufinden. Bei den Tieren der Gruppe II stiegen die Werte bis 1 Stunde nach der Eingabe an (1,44%) und fielen dann über 0,87% nach 2 Stunden, 0.76% nach 4 Stunden auf 0.75% nach 6 Stunden ab. Ab 4 Stunden nach der Eingabe waren die Werte in beiden Gruppen also praktisch gleich.

In Gruppe III war ein starker Abfall der Werte 30 Minuten nach der Eingabe zu verzeichnen, und zwar von 0.55% auf 0.07%. Danach stiegen sie leicht an und blieben ab 2 Stunden nach der Applikation auf etwa gleicher Höhe (0.31% nach 2 Stunden, 0.26% nach 4 Stunden und 0.29% nach 6 Stunden). Bis auf den ersten Meßzeitpunkt lagen die Werte der Tiere dieser Gruppe wiederum wesentlich unter den entsprechenden der beiden anderen Gruppen.

3.2.4. Tibia (s. Abb. 18)

In allen drei Gruppen stiegen die Werte von 15 Minuten nach der Applikation (bei den Tieren der Gruppen I, II und III wurden hier 0,20; 0.11 und 0,015% der eingegebenen Dosis wiedergefunden) bis zum Ende des Meßzeitraums nach 6 Stunden an. Die entsprechenden Werte lagen zu dieser Zeit in Gruppe I bei 1,28%, in Gruppe II bei 1,00% und in Gruppe III bei 0,17%. Auch hier waren keine nennenswerten Unterschiede zwischen den Werten der Tiere der Gruppen I und II festzustellen. Die Werte der Gruppe III fielen dagegen stark ab.

3.2.5. Muskel (s. Abb. 19)

In den untersuchten Muskelproben waren 15 Minuten nach der Applikation ebenso wie zu den späteren Meßzeitpunkten naturgemäß nur sehr geringe Aktivitäten festzustellen. Zwischen den Tieren der Gruppen I und II wurden zu diesem Zeitpunkt keine Unterschiede festgestellt, in beiden Fällen wurden 0.04% der eingegebenen Aktivität/g Muskel wiedergefunden. Dagegen fielen die Tiere der Gruppe III sehr stark ab, nur 0,005% der eingegebenen Dosis waren feststellbar. 30 Minuten nach der Eingabe wurden bei den Tieren der Gruppe I und III 0,02%, bei denen der Gruppe II dagegen 0,11% der applizierten Dosis wiedergefunden. Nach 1, 2 und 4 Stunden lagen die Werte bei den Tieren der Gruppe I bei 0.12%, 0,09% und 0.07% und waren damit zu den genannten Meßzeitpunkten etwa doppelt so hoch als bei den Tieren der Gruppen II und III. 6 Stunden nach der Applikation wurden die höchsten Werte bei den Tieren der Gruppe II festgestellt (0.09%).

Die Werte für die Tiere der Gruppe I blieben etwa auf gleicher Höhe im Vergleich zu denen 4 Stunden nach der Eingabe, während sie bei den Tieren der Gruppe III stark abfielen. Die höchsten Werte wurden in Gruppe I nach einer Stunde, in Gruppe II nach 30 Minuten und in Gruppe III nach 4 Stunden gemessen.

4. Diskussion der Ergebnisse

In allen drei Gruppen wurde schon nach 1–2 Stunden der Hauptanteil des Phosphors der entsprechenden Verbindungen, der sich noch im Inhalt des Verdauungskanals befand, im Dickdarminhalt gefunden. Bei den Tieren der Gruppen I und II blieb dann der Anteil von 2 Stunden nach der Infusion der Phosphatlösungen bis zum Ende des Meßzeitraums etwa auf gleicher Höhe (22,1%–26.4% bei den Tieren der Gruppe I bzw. 22.7%–24,4% bei denen der Gruppe II), während der Anteil der im Dickdarminhalt der Tiere der Gruppe III wiedergefundenen Aktivität laufend größer wurde – nach 6 Stunden wurden hier 44.6% der applizierten Menge wiedergefunden. Da nach den Untersuchungen von CRAMER [2] mehr als 90% des resorbierten Phosphors vom Dünndarm aufgenommen werden, zeigen schon diese Werte eine wesentlich schlechtere Verwertbarkeit des Phosphors aus Graham'schen Polyphosphat im Vergleich zu den beiden anderen Phosphaten durch das monogastrische Tier. Die wesentlich höheren Werte an wiedergefundener Aktivität (in Prozenten der applizierten Dosis) in den hinteren Wandabschnitten des Dünndarms bis 30 Minuten nach der Eingabe und die der Dickdarmwand bis 4 Stunden nach der Eingabe bei den Tieren der Gruppe III gegenüber denen der Gruppen I und II lassen auf eine mehr oder weniger feste Bindung der Polyphosphatmoleküle in bzw. an der Darmwand schließen, was auf Grund des chemischen Verhaltens der Polyphosphate als durchaus wahrscheinlich anzunehmen ist. Jedoch auf Grund der ungenügenden Spaltung in resorbierbare Bruchstücke können nur sehr geringe Anteile die Darmschranke passieren. Die

Zunahme der wiedergefundenen Aktivitätswerte im Dickdarminhalt und die Abnahme der entsprechenden Werte in der gesamten Darmwand wie bei den beiden anderen Gruppen von 1 Stunde nach der Infusion an zeigen, daß erhebliche Mengen wieder in das Darmlumen abgeschieden wurden.

Obwohl in diesem Versuch mit wesentlich niedrigeren Phosphorgaben als in unseren Bilanzversuchen (4), in der Gruppe III 17,7 mg P/100 g Körpermasse, gearbeitet wurde, reichte auch für diese Mengen die Hydrolysekapazität des Verdauungstraktes nicht aus, um ein annähernd gleiche Resorption wie in Gruppe I zu erhalten.

Dagegen zeigen die Werte, daß bei den eingegebenen Phosphormengen in Gruppe II (16,5 mg/100 g Körpermasse) der Phosphor des Pyrophosphats praktisch genauso ausgenutzt wurde wie der des Orthophosphats. Das bestätigt die Ergebnisse unserer Bilanzversuche, wo in Versuchsreihe I mit niedrigerer Phosphorvorgabe als in den beiden anderen Versuchsreihen (die allerdings mit 60 bis 70 mg Pyrophosphat-P-Gabe/Tier und Tag um ein Vielfaches über den in diesem Versuch applizierten Mengen lag) eine etwa gleiche relative P-Retention in der Pyrophosphatgruppe im Vergleich zur Orthophosphatgruppe erhalten wurde [4]. Die Pyrophosphathydrolysekapazität muß demnach einen beträchtlichen Umfang haben. Die innerhalb der Fehlergrenze in den einzelnen untersuchten Abschnitten des Verdauungstrakts bis auf wenige Ausnahmen praktisch gleichen Werte (wiedergefundene Aktivität in Prozenten eingegebener Dosis) bei einer 1,5fach höheren Gabe an Pyrophosphat-P/100 g Körpermasse gegenüber den Orthophosphattieren (16,5 mg gegenüber 11 mg/100 g Körpermasse) sprechen auch für eine erhebliche Säurehydrolyse im Magen. Zumindest dürfte in Übereinstimmung mit den Behauptungen STARKENSTEINS [6] die Hydrolyse des Pyrophosphates eingeleitet werden. Auch EBEL [3] kommt in seiner zusammenfassenden Arbeit zu dem gleichen Schluß.

Die in den untersuchten Organen bzw. Organteilen in den verschiedenen Zeitabständen wiedergefundenen Aktivitäten zeigen ebenfalls eine praktisch gleiche Ausnutzung von Ortho- und Pyrophosphat-P und eine wesentlich schlechtere Verwertung des Phosphors aus Graham'schen Polyphosphat an. Besonders deutlich kommt das bei Betrachtung der Werte der Tibia-Knochen in den verschiedenen Zeitabständen zum Ausdruck, während beim Muskel diese Unterschiede naturgemäß auf Grund des sehr geringen P-Einbaus nicht so in Erscheinung treten.

Unter der Voraussetzung, daß nur Orthophosphat in nennenswertem Umfang die Darmschranke passieren kann – eine Tatsache, die aus der vorliegenden Literatur eindeutig hervorgeht – zeigen die Ergebnisse, daß bei den in diesen Versuchen applizierten P-Mengen die Hydrolysekapazität des resorbierfähigen Teils des Darmtrakts der Ratte ausreicht, um eine gleiche Ausnutzung des Pyrophosphat-P im Vergleich zum Orthophosphat-P zu ermöglichen. Eine Ausnutzung des Phosphors aus Graham'schen Polyphosphat findet zwar statt, aber in einem gegenüber den erstgenannten Verbindungen wesentlich geringerem Umfang. Unsere Ergebnisse decken sich in dieser Hinsicht völlig mit denen von SCHREIER und NÖLLER [5], die an Ratten ebenfalls ^{32}P-markiertes Pyrophosphat und Graham'sches Polyphosphat verabreichten, die Tiere aber erst 12 bzw. 24 Stunden nach oraler Infusion töteten. Auch zu diesen Zeitpunkten waren die Impulsraten der

untersuchten Organe nach Pyrophosphatgabe um ein Vielfaches höher als nach Gabe von Graham'schen Polyphosphat.

Die Erklärung dafür kann nur in einer ungenügenden Hydrolyse der langen Phosphatketten zu resorbierbarem Orthophosphat gesucht werden.

Zumindest für das monogastrische Tier scheiden also die höher kondensierten Phosphate als Phosphorquellen in der Diät aus.

Zusammenfassung

Nüchterne Versuchsratten erhielten ^{32}P markiertes Ortho-, Pyro- und Polyphosphat oral verabreicht. Jeweils 2–4 Tiere einer jeden Gruppe töteten wir nach 15, 30, 60, 120, 240 und 360 Minuten und prüften den Verbleib der ^{32}P-Aktivität.

In allen drei Gruppen wurde schon nach 2 Stunden der Hauptteil des Phosphors der entsprechenden Verbindungen, der sich noch im Verdauungskanal befand, im Dickdarminhalt ermittelt (71–82%). Dieser Anteil erhöhte sich 6 Stunden nach der Applikation auf über 90%. Während jedoch die wiedergefundene Aktivität (in Prozenten der applizierten Dosis) bei den Tieren, die Ortho- und Pyrophosphat erhielten, von 2 Stunden nach der Eingabe der Lösungen an etwa auf gleicher Höhe blieb, stieg dieser Wert bei den Tieren, die Graham'sches Polyphosphat erhielten, weiterhin stark an. Sowohl diese Befunde, als auch die in den anderen untersuchten Organteilen zu den verschiedenen Zeiten wiedergefundenen Aktivitäten zeigen, daß bei den infundierten P-Mengen der Phosphor des Pyrophosphats ebensogut wie der des Orthophosphats von den Tieren genutzt wird. Der Phosphor des Graham'schen Polyphosphates wird dagegen auf Grund der zu geringen Hydrolysekapazität wesentlich schlechter verwertet.

Резюме

Опытные крысы получали в рот натощак меченые ^{32}P орто-, пиро- и полифосфаты. Из каждой группы последовательно убивались по 2–4 крысы через 15 минут, 30 минут, 60 минут, 120 минут, 240 минут, 360 минут, причем изучалось местонахождение активности ^{32}P.

Уже через два часа во всех трех группах главная часть фосфора соответствующих соединений, еще находившаяся в пищеварительном канале, была обнаружена в содержимом толстых кишек (71–82%). Спустя 6 часов после скармливания эта доля увеличилась и составляла свыше 90%. Но в то время как у животных, получавших орто- и пирофосфаты, установленная активность (в процентах от примененной дозы) спустя два часа после дачи растворов оставалась на одинаковой высоте, у животных, получавших полифосфат Грахам'а, эта величина повышалась и дальше. Как эти данные, так и установленные в различное время в других исследовавшихся частях тела величины активности показывают, что при скармливавшихся количествах P фосфор пирофосфата используется

животными так же хорошо, как и фосфор ортофосфата. Фосфор же полифосфата Грахам'а, вследствие слишком малой емкости гидролиза, используется ими значительно хуже.

Summary

Fasting experimental rats received oral doses of ^{32}P-labelled ortho-, pyro- and polyphosphate. 2–4 animals of each group were killed after 15, 30, 60, 120, 240 and 360 min tested for the ^{32}P activity.

As early as after 2 hours the bulk of the phosphorus of the corresponding compounds present in the alimentary canal was recovered from the large intestine (71–82%) in all 3 groups of animals. 6 hours after administration the proportion exceeded 90%. In animals receiving ortho- and pyrophosphate the amount of radioactivity recovered (expressed as percentage of the dosis applied) remained at about the same level, from 2 hours after injection of the solution onwards, whereas in animals receiving the Graham polyphosphate this figure kept rising. These data as well as the data for the amount of radioactivity recovered at different times in other parts of the organs tested indicate that with the amounts of P ingested the animals were able to utilise phosphorus from the pyrophosphate as effectively as phosphorus from the orthophosphate. Phosphorus from Graham polyphosphate, however, is less readily utilised due to its low capacity for hydrolysis.

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in farmyard manure or fertilisers; 1 ton/acre supplies 270 lb. of Mg/acre. Information on the availability to crops of Mg in this form is limited; Wolton[4] reported that over a short period it was less efficient than equivalent rates of Mg as sulphate in raising the Mg content of herbage. This may be because of lower solubility or because of the antagonistic effect of Ca. In one experiment on potatoes, each of these sources supplying 27 lb. of Mg/acre gave the same increases in yield. At the high rates of magnesian limestone normally applied, however, it is probable that even with slower availability sufficient Mg would be supplied to the following crop.

Mg deficiency, serious enough to be economically important, seems therefore to be restricted to soils 'low' or 'very low' in exchangeable Mg, even among light soils these are not common. Even on deficient soils the deficiency is often corrected in practice by farmyard manure or fertilisers containing Mg. Serious deficiency can be overcome by liming with magnesian limestone instead of liming materials containing little or no Mg. Where this is too expensive because of high transport costs, or where liming is not needed, 30–40 lb. of Mg/acre can be supplied as the sulphate (either kieserite or Epsom salts) or as calcined magnesite, given every 3 years or so. While there is inadequate information on the necessary rate of frequency of application of Mg, it seems advisable to apply it in relatively small and frequent applications because of the risk of loss by leaching.

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BIOLOGICAL EFFECTS OF FOOD ADDITIVES. II.*—Sodium Pyrophosphate

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The acute effects of oral and parenteral sodium pyrophosphate on rats have been examined, with determination of the intraperitoneal LD_{50} and the cause of death.

Sodium pyrophosphate was included for 16 weeks at 1%, 2½% and 5% levels and sodium orthophosphate at a 5% level in the diet of rats. The effects were assessed by examining gain in body weight, food intake, renal and hepatic function, blood cell counts, organ weights and gross pathology and micropathology. Calcium and phosphorus balance studies were carried out on some groups. Faeces and urine have been analysed for content of pyrophosphate.

The intraperitoneal LD_{50} of sodium pyrophosphate was 233 mg./kg., whereas the oral LD_{50} was probably greater than 4 g./kg. The cause of death was not established.

The low oral acute toxicity, the balance studies and the analyses of faeces and urine all suggest that pyrophosphate is converted to orthophosphate in the body. The site of such a reaction is being further studied.

The longer term studies indicate that a 1% level of pyrophosphate in the diet of rats caused renal damage. The relevance of the finding to the ingestion of phosphate by man is discussed. Further studies are desirable on the metabolism of pyrophosphate and on the assessment of a safe phosphate intake for man.

Introduction

At the present time the use of phosphoric acid and its simple and condensed salts in food

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is increasing.[1] The pure acid is used extensively, instead of citric or tartaric acid, in a certain type of soft drink[2] and the salts are used as sequestering agents, emulsifiers, texturisers and buffers in a wide range of food products.[1, 3]

It has long been known that orthophosphates may cause renal damage when fed to rats.[4] Van Genderen and co-workers[5] observed that both orthophosphates and a mixture of long-chain polyphosphates and pyrophosphates caused a deposition of calcium phosphate in rat kidneys without indication of tissue damage, when present at a 0·5% level in their diet (equivalent to 0·3 g./kg. body weight/day). With diets containing 1% of either preparation (0·6 g./kg./day), formation of concretions was more pronounced, with evidence of tissue damage. Hodge[6] reported that in short-term toxicity tests on rats, condensed phosphates caused kidney damage when included at 2% level in the diet (1·2 g./kg./day). No significant abnormalities were seen in the kidneys of rats receiving a diet containing only 0·2% (0·12 g./kg./day). Dogs fed on the phosphates showed no deleterious effect at the 0·1 g./kg. body weight/day level, but damage was obvious at the 4 g./kg./day level.

In view of the present-day use of pyrophosphates in baking powder, liquid foods, chocolate-drink powders, soup thickeners, instant puddings, ice cream, meat and cheese and also of the effects of the sodium salt when administered parenterally to mice, rabbits and dogs,[7] further examination of the acute effects of parenteral administration and the acute and longer-term effects of oral administration of sodium pyrophosphate seemed desirable. This paper describes such an investigation. For administrative reasons outside our control it was not possible in these studies to carry out life-span tests; however, since pyrophosphate is a normal body constituent, life-span studies at low doses may be less important than the acute and medium-term effects of relatively high dosage levels.

Experimental

Materials

Sodium pyrophosphate.—Supplied by Messrs. Albright & Wilson, as Tetron K, analysing as 97·5% sodium pyrophosphate and 2·5% orthophosphate.

Disodium hydrogen phosphate.—Grade used in food manufacture, supplied by Messrs. Albright & Wilson; containing 39·5–40·0% P_2O_5.

Diets.—The composition of the five diets is shown in Table I. They were prepared in 3 kg. batches, as required, and stored in covered tins in a cool room.

Table I

Composition and analysis of diets

(weight of constituent in g. of diet)

Constituent	Control	1% Pyro-phosphate	2½% Pyro-phosphate	5% Pyro-phosphate	5% Ortho-phosphate
Whole wheat flour	660·0	660·0	643·0	615·0	615·0
Full cream dried milk	330·0	330·0	330·0	330·0	330·0
Salt mix	10·0	—	—	—	—
Casein	—	—	2·0	5·0	5·0
Sodium pyrophosphate	—	10·0	25·0	50·0	—
Disodium hydrogen phosphate dihydrate	—	—	—	—	62·7
Total	1000·0	1000·0	1000·0	1000·0	1012·7
Calorific value, cal./100 g. (calculated)	397	397	398	399	394
Protein content, g./100 g. diet (calc.)	17·2	17·2	17·1	17·1	17·1
Calcium, mg./100 g. diet (determined)	644	—	—	448	446
Phosphorus, mg./100 g. diet (determined)	526	—	—	1650	1598

Animals.—The rats used were from our own breeding stock of Birmingham-Wistar strain.

Dosage and relationship to level of use

The possible daily consumption of sodium pyrophosphate by a 70-kg. man has been calculated from the values in Table II as 423 mg./day (6·03 mg./kg./day). This is the standard daily dietary dose *D* for man.

Table II

Pyrophosphate content of foods and possible daily human intake

Foodstuff	Max. % concn. of pyrophosphate*	Foodstuff intake, g./day[b]	Consumption of pyrophosphate, mg./day
Processed cheese	3·0	1·5	45
Cured meats	0·5	4·9	25
Sausage	0·5	14·2	71
Ice cream	0·05	28[c]	14
Flour (self-raising)	0·45	28·8	130
Cheese, scones, etc.	0·25*	23·4	58
Instant pudding	2·0	4·0[c]	80
		Total	423 mg.

* From Publ. 398, Nat. Res. Coun., Wash., 1956
[b] From Domestic Food Consumption and Expenditure, 1957 (London: H.M.S.O.)
[c] Estimated

The concentration needed in the diet to supply this dosage level to rats has been calculated, assuming that the mean body weight of animals is 250 g. and that they eat 15 g. of food each day. A 250 g. rat must consume 1·508 mg. to receive a dose equivalent to D on a mg./kg. body weight basis. This in 15 g. of diet = 100 mg./kg. diet, approximately. Hence a diet containing 10 g./kg. would supply rats with the equivalent of approx. $100 \times D$. It is emphasised that this is an approximate figure only. An independent estimate set the mean intake in England to be 300 mg. per person, but the figure must vary considerably from person to person.

Acute toxicity

The acute effects of parenteral administration were examined in order to determine whether the biological activity of pyrophosphate was altered by passage through the wall of the gastrointestinal tract. This might be important in those suffering from gastrointestinal lesions, in whom the salt could be absorbed directly into the blood without passing through the gastrointestinal barrier.

Table III

Intraperitoneal dosage levels of sodium pyrophosphate in LD_{50} determination

Dose, mg./kg. body weight	Number of rats	Number dying in 16 h.
1000	6	6
631	6	6
398*	6	6
251*	6	4
159*	6	0
100*	6	0
63	2	0
40	2	0
25	2	0

* Used for calculation of median lethal dose (LD_{50})

An aqueous solution of sodium pyrophosphate was administered by intraperitoneal injection to male rats weighing between 170 and 210 g., at the dosage levels shown in Table III. The solution was made by dissolving 10 g. of sodium pyrophosphate in 400 ml. of distilled water, adjusting the pH to 7·2 with hydrochloric acid, and making up the volume to 500 ml. with water. The animals were observed for 10 days; all were autopsied.

A 3% aqueous solution of sodium pyrophosphate was administered orally to 10 rats at the 1600 mg./kg. dosage level. They were killed after 10 days and autopsied.

The possibilities that death following parenteral administration was caused by hypocalcaemia, the osmotic effect of the solution, alkalosis, or by an effect on respiratory enzymes were examined.

Sodium pyrophosphate was administered to 4 rats at the 800 mg./kg. level and serum calcium and packed cell volume determinations were made after 1 h.

Sodium chloride, in solution of similar osmolarity to sodium pyrophosphate at the 1600 mg./kg. dosage level, was administered by intraperitoneal injection to 4 rats.

Sodium bicarbonate (2020 mg./kg.), a dose containing the same number of sodium ions as sodium pyrophosphate at the 1600 mg./kg. dosage level, was injected intraperitoneally into 4 rats.

The respiration rate of rat liver slices and rat liver homogenate in isotonic sucrose solution

was determined by the Warburg technique in the presence of sodium pyrophosphate and sodium orthophosphate (pH adjusted to 7·4) up to a final concentration of 0·1% of the salts.

The action of pyrophosphate solution on the intestine and on skin was observed. Two rats injected with the 2% solution of sodium pyrophosphate at the 400 mg./kg. dosage level, two with sodium orthophosphate at the same level and two with normal saline, were killed and examined 1 h. after the injection. A series of concentrations of sodium pyrophosphate and orthophosphate (all adjusted to pH 7·4) ranging from 0·5% to 4·0% was injected intradermally into the shaved backs of rats.

Short term toxicity

(1) *Pilot experiment*

Three groups of 10 rats were given diets containing (*a*) 10% sodium pyrophosphate, (*b*) 5% sodium pyrophosphate and (*c*) no sodium pyrophosphate. The basal diet was a flour/dried milk mixture similar to that described in Table I. The growth rate and food intake of each group were measured daily. It was found that in 2 weeks, the rats with the diet containing 10% sodium pyrophosphate lost weight and 5 died. Their food intake was lower than that for the control group. The rats given 5% sodium pyrophosphate in their diet ate less than the control animals and grew at a slower rate, but appeared normal in all other ways. It was decided to feed a maximum of 5% of the salt in the diet in the longer-term feeding test, since the 10% level was not tolerated.

(2) *General plan of main experiment*

Five groups of rats, each group consisting of 20 male and 20 female animals weighing between 90 and 115 g., were used. Ten males and ten female animals of each group were caged individually and the remainder were kept five to a cage. All animals were housed in a room at 72° ± 2° F under similar conditions of relative humidity and light availability. All were allowed drinking water *ad libitum*.

In this text, the diets will be referred to as control, 1%, 2½% and 5% pyrophosphate and 5% orthophosphate diets, and the animal groups will be referred to as C, 1% PP, 2½% PP, and 5% PP and 5% OP groups respectively.

The weekly weight gain and food intake were determined to the nearest g. for 100 days on each rat caged individually.

After 3 weeks on the diet the food consumption was determined and the faeces and urine collected from 5 male animals each of the C group and of the 5% PP group during a 6-day period. A similar collection was carried out after 8 weeks on 5 male animals each of the 5% PP and the 5% OP groups during a 3-day period. Food, urine and faeces were analysed for calcium by the flame photometric method, for phosphorus by the molybdate colorimetric method and for pyrophosphate by the method described below.

Determination of pyrophosphate in faecal matter and urine

Principle.—The method was based on the observation of Flynn *et al.*[8] that pyrophosphate does not normally give any colour with the molybdate reagent of Fiske & Subbarow, but develops a blue colour when cysteine is added.

Technique.—Total faecal matter was homogenised and made up to 500 ml. Ten ml. of this homogenate were mixed with 10 ml. of perchloric acid (30%), diluted to 100 ml. and filtered. Two ml. of this filtrate were taken in a 25-ml. volumetric flask and diluted to about 20 ml. There were then added 2·0 ml. of molybdate reagent, 1 ml. of cysteine solution and finally 1 ml. of aminonaphtholsulphonic acid (A.N.S.) reagent. (The molybdate reagent, cysteine solution and A.N.S. reagent were prepared as described by Flynn *et al.*[8]) The volume was made up to 25 ml. and the optical density (O.D.) of the blue solution measured at 660 mμ. after 7 min.

Two standards were set up as follows: (i) 2 ml. of orthophosphate solution, containing 20 μg. of P; (ii) 2 ml. of pyrophosphate solution, containing 10 μg. of P, and treated exactly in the same manner. Two ml. of water with all the reagents added as above were taken as a blank. After exactly 3 h. the optical densities at 660 mμ. of all the test and standard solutions were again measured.

Calculation

Total pyrophosphate in sample (as P) =

$$\frac{D_2 - (D_1 \times O_2)/O_1}{P_2 - P_1} \times 10 \times \frac{100}{2} \times \frac{500}{10}\ \mu g. = \frac{D_2 - (D_1 \times O_2)/O_1}{P_2 - P_1} \times 25\ mg.$$

where D_1 and D_2 = O.D. of test solution after 7 min. and 3 h. respectively
O_1 and O_2 = O.D. of orthophosphate standard after 7 min. and 3 h. respectively
P_1 and P_2 = O.D. of pyrophosphate standard after 7 min. and 3 h. respectively

Function tests

(a) *Liver function.*—The bromosulphalein (BSP) clearance test was performed on 5 male and 5 female rats of each group after 110 days. Each rat received 25 mg./kg. body weight of BSP in saline solution by intravenous injection. The dye remaining in the blood after 20 min. was determined colorimetrically (Ahmad & Frazer[9]).

(b) *Kidney function.*—The phenol red excretion test and the concentration test were carried out as described by Sharratt.[10] In the former the percentage of dye excreted in the urine after an intramuscular injection of the dye was determined, and in the latter the specific gravity of urine passed by the animals between 8 and 24 h. after deprivation of water was measured. The albumin and cellular contents of this urine were examined.

Haematology

The usual methods were employed to determine the red cell, white cell and haemoglobin contents of venous blood of 5 male and 5 female rats of each group at the end of the experiment.

Organ weight

The animals living in individual cages were killed by exsanguination under ether anaesthesia. Various organs were removed from the rats, trimmed of fat and adherent tissues and weighed immediately. Organ weights were expressed as mg. of organ/100 g. body weight.

Autopsy

During the determination of organ weights, the organs were examined macroscopically for pathological abnormalities. Autopsies were also carried out on the rats kept 5 to a cage at the end of the experiment.

Histology

The organs were fixed in formol saline, embedded in paraffin and stained with haematoxylin and eosin.

Results

(1) *Acute toxicity*

The median lethal intraperitoneal dose (LD_{50}) of pyrophosphate, calculated by the method of Weil[11] from the figures marked with an asterisk in Table III, was 233 mg./kg. body weight, with 95% confidence that the true value lay between 192 and 282 mg. kg. body weight.

Rats receiving sodium pyrophosphate intraperitoneally at the highest levels became gradually weaker and insensitive to pain and died within 2 h. They did not lose consciousness or the corneal reflex before death. The breathing of many became shallower and slower and some had slow gasping respiration.

Rats receiving lower doses behaved similarly. One of the group receiving 398 mg./kg. body weight died within 2 h. and the rest of the group between 2 and 16 h. All animals that died did so within 16 h.

No effects were seen in the rats receiving doses of 25 and 40 mg./kg., but those receiving 63 mg./kg. became weaker and more reluctant to move than those on the lower doses. Rats receiving 100 mg./kg. were markedly weaker and several showed a gasping type of respiration.

Rats dying within 2 h. showed, post-mortem, slight redness of the serous surface of the small

intestine and fluid in the peritoneal cavity. Nothing abnormal was detected in rats killed after 10 days. The intestines of rats killed 1 h. after injection of sodium orthophosphate or of saline appeared to be normal and responded to a pinprick by contracting. The intestines of rats killed 1 h. after an injection of pyrophosphate showed areas of hyperaemia on the surface and these parts did not respond to a pinprick, although other parts of the intestines with normal appearance did.

Fluid from the peritoneal cavity of the rats receiving pyrophosphate contained many red, white and epithelial cells and a considerable concentration of coagulable protein. Fluid from control rats contained no red cells, a few white and epithelial cells and much less coagulable protein. Examination of the blood of rats receiving sodium pyrophosphate at the 800 mg./kg. level showed that their serum calcium was reduced from the normal mean level of 9·4 mg.-% (range 9·0–9·6) to 5·1 mg.-% (range 4·4–6·0). Rats receiving sodium orthophosphate at the same dosage level had a serum calcium level of 7·4 mg.-% (range 7·2–7·6). However, the administration of massive doses of calcium gluconate had no therapeutic effect on animals receiving sodium pyrophosphate. There was marked haemoconcentration in these animals (packed cell volume = approx. 75%, control approx. 50%).

Rats receiving sodium chloride of similar osmolarity, or sodium bicarbonate of similar sodium ion content, to that of sodium pyrophosphate at the 1600 mg./kg. dosage level showed no untoward effects.

Tissue respiration studies showed that concentrations of up to 0·1% of sodium orthophosphate or pyrophosphate did not significantly increase or decrease the respiration rate of liver from its normal value of 2·14 (range 1·47–3·51) μl. of O_2/mg. of dry tissue/h.

Intradermal administration of the phosphates showed that concentrations up to 4% of sodium orthophosphate had only a slight reddening effect on the skin. Solutions of sodium pyrophosphate, 1% or stronger, caused necrosis of the injected area; a 0·5% solution had an effect similar to that of 4% sodium orthophosphate.

(2) *Short term toxicity*

Weight gain and food intake

The mean gain in body weight and mean cumulative food intake of rats of each group (Table IV) show that male and female rats fed on the 5% pyrophosphate diet grew at a slower rate and had a lower food intake than other groups.

Table IV

Mean body weight gains and food intakes of rats fed on diets containing pyrophosphate or orthophosphate

(Mean weight gains and standard errors are in g.)

Group	Males		Females	
	Body weight gain	Food intake	Body weight gain	Food intake
Control	210 ± 20	1309 ± 28	137 ± 7	1184 ± 28
1% Pyrophosphate	245 ± 16	1359 ± 27	129 ± 6	1110 ± 28
2½% Pyrophosphate	229 ± 15	1305 ± 24	129 ± 7	1116 ± 16
5% Pyrophosphate	187 ± 15	1213 ± 48	114 ± 7*	1055 ± 26
5% Orthophosphate	234 ± 14	1363 ± 18	140 ± 8	1168 ± 23

* Significantly different from control group $P < 0·05$.

The significance of the difference between the 5% PP group males and the other groups was obscured by the slow growth rate of the male control group. These grew at a much slower rate than the 1% and 2½% PP and the 5% OP groups, and control animals of the same stock used in other experiments. The growth rate of our control animals usually follows closely the growth rate of the 1% PP group.

To determine whether the decreased food intake of male and female rats of the 5% PP group would account completely for the decrease in their growth rate, an analysis of covariance of food intake and gain in body weight of each group was made by the method of Crampton.[12]

This analysis divides the food intake into two parts: (1) the food used for each gramme of body weight gained, and (2) the food used to maintain the body at constant weight. The results showed that the amount of food used for unit gain in body weight and maintenance by the males and females fed on the 5% pyrophosphate diet was not significantly different from that used for these purposes by the other groups.

Balance studies

The results of the analyses of food, urine and faeces, summarised in Table V, show that sodium pyrophosphate and orthophosphate are absorbed and excreted and affect calcium metabolism in a similar manner. The pH of urines passed by control, 1% and 2½% PP groups were not significantly different from each other (pH 6·7), but were significantly less alkaline than urine from the 5% PP and 5% OP groups (mean of both = pH 7·5). There were no differences in the concentrations of sodium, potassium or ammonium in the urine of any group. Such similarity suggested that pyrophosphate may be hydrolysed to orthophosphate before or during absorption in the rat gut. This was substantiated by the fact that pyrophosphate could never be detected in the faeces or urine of animals receiving the salt. We have found that rat faeces are capable of hydrolysing pyrophosphate to orthophosphate.

Table V also shows that pyrophosphate was almost completely absorbed by the gut and excreted by the kidneys as orthophosphate. The Ca/P balance was upset by loss of weight in 2 rats of the 5% PP group, when this and the control group were examined, but these 2 rats also showed the almost complete absorption of pyrophosphate.

Table V

Total phosphorus (phosphate and pyrophosphate) and calcium eaten, and % phosphorus (phosphate only, no pyrophosphate found) and calcium excreted and retained by rats fed on basal diet or on a diet containing orthophosphate or pyrophosphate

	Calcium				Phosphorus				mg. Ca retained / mg. P retained	Body weight gain, g.
	mg. eaten	% excreted in faeces	% excreted in urine	% retained	mg. eaten	% excreted in faeces	% excreted in urine	% retained		
Control	99·9 ± 13·86	48·7 ± 0·89	0·65 ± 0·05	51·3 ± 0·92	81·7 ± 11·35	60·1 ± 1·13	11·4 ± 0·18	28·5 ± 1·18	2·2 ± 0·09	3·3 ± 1·1
5% Pyrophosphate	63·3 ± 4·51	43·6 ± 1·95	1·77 ± 0·06	54·7 ± 1·95	236·6 ± 18·76	16·5 ± 0·73	82·5 ± 3·12	0·86 ± 2·60	4·11 ± 0·82	0·5 ± 0·5
5% Orthophosphate	59·2 ± 1·57	47·4 ± 0·74	0·79 ± 0·10	51·8 ± 0·78	211·6 ± 5·73	15·2 ± 1·21	75·2 ± 0·93	9·6 ± 1·27	1·6 ± 0·18	4·1 ± 0·84
5% Pyrophosphate	56·3 ± 2·24	43·9 ± 2·57	1·06 ± 0·06	55·0 ± 2·58	206·6 ± 8·29	14·9 ± 0·78	74·2 ± 3·03	10·9 ± 2·86	2·9 ± 1·47	3·9 ± 0·51

The figures are the mean and standard error of 5 animals from each group

Function tests

The results of the liver and kidney function tests (Table VI) show that liver function, as estimated by the BSP clearance, was not different in experimental and control animals. Of the kidney function tests, the phenol red test was very variable in the males. The females of the 5% PP and 5% OP groups excreted the dye at a slower rate than normal, but the retardation did not reach a statistically significant level.

The concentration tests showed that males of the 2½% PP, 5% PP and 5% OP groups and females of the 5% PP and 5% OP groups had impaired renal function. There was no significant increase in urine albumin or cell content in any group.

Haematology

The results (Table VII) show that although there were some significant differences between certain counts, there were no significant trends towards abnormality as the concentration of pyrophosphate was increased.

Table VI

Liver and kidney function of rats fed diets containing pyrophosphate or orthophosphate

Group	Liver function, mg. of BSP/100 ml. serum after 20 min.	Kidney function: Phenol red test (% excreted in ½ h.)	Kidney function: Concentration test (sp. gr.)
Males			
Control	0·53 ± 0·09	16·72 ± 2·11	1·0622 ± 0·0026
1% Pyrophosphate	0·43 ± 0·14	9·64 ± 2·36	1·0547 ± 0·0047
2½% "	0·63 ± 0·12	15·32 ± 4·07	1·0429 ± 0·0061*
5% "	0·37 ± 0·12	16·10 ± 5·59	1·0432 ± 0·0051*
5% Orthophosphate	0·65 ± 0·10	12·74 ± 2·30	1·0488 ± 0·0013**
Females			
Control	0·55 ± 0·20	36·94 ± 2·66	1·0592 ± 0·0024
1% Pyrophosphate	0·55 ± 0·09	35·55 ± 2·26	1·0628 ± 0·0026
2½% "	0·42 ± 0·14	40·63 ± 4·36	1·0561 ± 0·0015
5% "	0·39 ± 0·13	27·62 ± 5·78	1·0445 ± 0·0014**
5% Orthophosphate	0·38 ± 0·15	29·78 ± 2·78	1·0513 ± 0·0025*

* Significantly different from control group $P < 0·05$
** Significantly different from control group $P < 0·01$

Table VII

Haematological findings in rats fed on diets containing pyrophosphate or orthophosphate

Group	Red blood cells, millions/cu.mm.	Haemoglobin, g./100 ml.	Lymphocytes per cu.mm.	Granulocytes per cu.mm.	Monocytes per cu.mm.
Males					
Control	7·51 ± 0·20	13·1 ± 0·17	10,968 ± 1767	2664 ± 513	908 ± 204
1% Pyrophosphate	7·95 ± 0·25	12·8 ± 0·24	6324 ± 455*	2511 ± 504	995 ± 97
2½% "	7·75 ± 0·17	12·4 ± 0·21*	10,080 ± 1720	2787 ± 619	723 ± 226
5% "	7·67 ± 0·18	12·5 ± 0·25	14,839 ± 3413	4139 ± 1328	1242 ± 377
5% Orthophosphate	8·25 ± 0·31	13·9 ± 0·29*	8759 ± 1485	2686 ± 358	265 ± 96*
Females					
Control	7·30 ± 0·18	12·4 ± 0·51	11,204 ± 1871	1875 ± 258	361 ± 98
1% Pyrophosphate	7·21 ± 0·15	12·0 ± 0·46	8051 ± 1366	1723 ± 70	242 ± 96
2½% "	7·00 ± 0·19	12·9 ± 0·23	11,318 ± 1597	2449 ± 771	544 ± 138
5% "	6·91 ± 0·53	12·1 ± 0·36	7384 ± 563	4275 ± 1084	572 ± 167
5% Orthophosphate	7·92 ± 0·13*	13·2 ± 0·54*	9557 ± 1342	2201 ± 1285	611 ± 408

* Significantly different from control group $P < 0·05$

Table VIII

Organ weights of rats fed on diets containing pyrophosphate or orthophosphate

(weights are mg./100 g. live weight ± standard error)

Organ	Control	1% Pyrophosphate	2½% Pyrophosphate	5% Pyrophosphate	5% Orthophosphate
Males					
Heart	244 ± 4	255 ± 6	251 ± 77	295 ± 19*	256 ± 7
Liver	3170 ± 133	3097 ± 75	3029 ± 87	3452 ± 234	3112 ± 57
Spleen	271 ± 14	233 ± 7	261 ± 16	286 ± 15	256 ± 13
Stomach	378 ± 12	365 ± 15	388 ± 12	608 ± 39**	407 ± 14
Intestine	2229 ± 112	2108 ± 111	1964 ± 83	2716 ± 239	1940 ± 99
Adrenals	13 ± 1	11 ± 0·6	12 ± 0·9	13 ± 0·7	12 ± 0·4
Kidneys	634 ± 21	598 ± 17	648 ± 24	769 ± 34*	741 ± 15**
Testes	962 ± 42	900 ± 29	905 ± 42	1233 ± 79*	1005 ± 37
Females					
Heart	278 ± 4	273 ± 5	295 ± 10	311 ± 9*	285 ± 9
Liver	3146 ± 33	3045 ± 80	3048 ± 129	3292 ± 92	3021 ± 65
Spleen	297 ± 14	304 ± 24	305 ± 20	334 ± 20	285 ± 30
Stomach	451 ± 16	459 ± 15	500 ± 22	721 ± 63**	486 ± 23
Intestine	2521 ± 73	2928 ± 149	2626 ± 85	3203 ± 149**	2696 ± 103
Adrenals	27 ± 1·5	26 ± 1·7	26 ± 1·0	26 ± 1·7	25 ± 2·0
Kidneys	601 ± 12	629 ± 21	743 ± 24**	893 ± 42**	838 ± 35**

* Significantly different from control group $P < 0·05$
** Significantly different from control group $P < 0·01$

Organ weights

The relative organ weights are shown in Table VIII. It is seen that rats of the 5% PP group had increased relative weights of heart, liver, spleen, stomach, intestines, kidney and testes. Of these, in comparison with the control group, the relative weights of heart, stomach, intestines (females only), kidneys and testes reached a high level of significance. Increases in relative weight were not found in other groups, except for intestines in females of the 1% PP group and kidneys in the 5% OP (males) group and 2½% PP and 5% OP (females) groups. The females of the 1% PP group had smaller spleens than normal, but no reason for this could be established.

Differences in relative heart weight between control and 5% PP groups could be accounted for to a large extent by the difference in body weight of the groups, but differences in body weight could not account entirely for differences noted in weight of the other organs.

Autopsy and histology

The results of macroscopic examination of the organs of rats kept individually in cages are shown in Table IX. Apart from these, no other abnormalities were noted. Of the organs examined, only the kidneys showed micropathological changes. The stomachs of the 2½% PP and 5% PP groups appeared to be microscopically normal.

Table IX

Appearance of organs on autopsy of rats fed on diets containing pyrophosphate or orthophosphate

Appearance of organ	Number of each group with abnormality (10 animals/group)									
	Male					Female				
	Control	Pyrophosphate			Orthophosphate	Control	Pyrophosphate			Orthophosphate
		1%	2½%	5%	5%		1%	2½%	5%	5%
Pale, pitted kidneys	—	—	—	6	6	—	—	2	5	7
Calcification of kidneys (macroscopic)	—	—	—	5	6	—	—	2	5	5
Hypertrophy of cardiac/pyloric border of stomach	—	—	—	4	—	—	—	3	6	—
Haemorrhages at this site	—	—	—	4	—	—	—	3	4	—

Renal pathology is summarised in Table X. It is seen that with the lower doses of pyrophosphate, the changes were chiefly in the cortex. The medullary zone was more affected in rats receiving the high concentrations of pyrophosphate and orthophosphate.

Table X

Microscopical appearance of kidneys of rats fed on diets containing pyrophosphate or orthophosphate

	% of each group with abnormality				
	Control	Pyrophosphate			Orthophosphate
		1%	2½%	5%	5%
Renal damage	25	95	100	100	100
Cortical atrophy	15	60	60	15	15
Cortical hyaline degeneration	10	55	55	20	10
Cortical calcification	0	5	10	10	5
Medullary calcification	0	5	30	70	75
Medullary necrosis	0	5	10	60	65
Tubular casts	5	0	30	55	55
Haemorrhages and exudate	5	45	50	60	60
Chronic inflammatory changes	0	0	5	70	70

Discussion

The acute studies show marked differences between oral and parenteral LD_{50} levels. The oral figure is in the region of 4 g./kg., whereas the parenteral is less than 250 mg./kg. body weight. Death on intraperitoneal injection was probably due to effects that are unlikely to occur after oral administration. The difference between oral and parenteral toxicity may also be related to the conversion of pyrophosphate into orthophosphate, since the orthophosphate has much

less effect when administered parenterally. The site and mechanism of this conversion is being further investigated; it is generally thought that it occurs in the gastrointestinal tract during absorption. Whether the effects observed on cells may have some significance in patients who have had a gastrectomy may be worthy of further study.

When pyrophosphate was fed at a level of 2½% or 5% of the diet, certain effects were observed. At the highest level there was some interference with weight gain. Although this could be accounted for by reduction in food intake, it should, perhaps, be regarded as an indication of a toxic effect. This matter is being further studied, although in this particular investigation the point was not of major importance since other well-defined effects were also present.

Pyrophosphate administration at a level of 5% of the diet caused a significant increase in the relative weight of the small intestine which was not observed when a similar concentration of orthophosphate was fed. This may be related to the metabolic handling of the pyrophosphate; however, the effect was also observed in the stomach, which suggested a slight irritant effect at this level of administration.

The kidney weight was significantly increased and renal function impaired in the groups receiving 2½% and 5% of pyrophosphate and in the group having 5% of orthophosphate, but no difference from the controls was observed in the 1% pyrophosphate group. However, histopathological studies revealed renal damage in all these groups including the 1% level.

These studies show that the administration to rats of pyrophosphate at a concentration of 1% or more in the diet or of orthophosphate at a 5% level resulted in demonstrable changes in the kidneys which showed reduced functional efficiency and increased relative weight. In these studies lower levels of orthophosphate were not fed, but this has been done by others.[4,5]

The clearance level for pyrophosphate on this basis, so far as the rat is concerned and assuming the usually accepted margin of safety of 100×, would be 0·01% in the diet, which for an adult rat corresponds to 6 mg./kg. body weight/day.

There is always some doubt about the validity of transferring information obtained from animals to human subjects and this may be important here, since the rat kidney has exceptional powers of concentration and may be more susceptible to damage from phosphate than kidney from other species. It may also be questioned whether the safety margin of 100× is not unnecessarily low for normal body constituents, such as phosphate. However, if the transference is made, it is apparent that the human daily intake cleared on the basis of these rat studies and a margin of safety of 100× is in the region of 420 mg./day for an adult man of 70 kg. body weight. This is close to the average daily intake of pyrophosphate calculated earlier in this paper. Since pyrophosphate appears to be converted to orthophosphate, it would seem advisable to consider the total phosphate load, which must be considerably greater than the calculated figure for pyrophosphate used as a food additive.

Although there is no indication that present dietary phosphate loads are excessive, these studies suggest that some further investigation of this problem is desirable. In particular, further information is needed on the mechanism and site of conversion of pyrophosphate to orthophosphate, on the toxicological significance of the observed renal changes, on the mechanism by which they are brought about and the extent to which the rat may be particularly susceptible to such changes. From these further investigations, it should be possible to make some assessment of the acceptable limits of phosphate intake for man.

Acknowledgments

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AMMONIA LOSS FROM AMMONIACAL FERTILISERS APPLIED TO CALCAREOUS SOILS

By S. LARSEN and D. GUNARY

Ammonia volatilised from ammonium salts applied to calcareous soils was greatest from ammonium sulphate (AS), about half as much from mono- and di-ammonium phosphates (MAP and DAP) and ammonium nitrate (AN), and negligible from magnesium ammonium phosphate.

Losses of ammonia in 8 days from ammonium salts applied to an initially acid, sandy soil treated with calcium, barium or magnesium carbonates, were: soil treated with $CaCO_3$, AS ≫ DAP = AN; with $BaCO_3$, AN ≫ AS = DAP; with $MgCO_3$, AS > AN ≫ DAP.

Reasons for these findings are discussed. It is evident that factors other than pH are operating.

Introduction

Since the use of chemical fertilisers began more than 100 years ago, it has been recognised that nitrogen can be lost as gaseous ammonia when an ammoniacal fertiliser is applied to calcareous soil. Recent studies, e.g., by Steenbjerg[1] and Jensen & Kjaer,[2] have confirmed this. Steenbjerg presumed that experimental results obtained with ammonium sulphate were also applicable to other ammonia salts. van Schreven,[3] however, found that the rate of loss of ammonia by volatilisation from ammonium salts applied to calcareous soils was dependent on the anion present in the salt. Loss from ammonium sulphate was found to be more rapid than from ammonium nitrate and this was confirmed by Nommik & Warlin.[4] As the loss of ammonia depends on the equilibrium $NH_4^+ \rightleftharpoons NH_3 + H^+$, van Schreven[3] considered that the difference in the rates of loss of ammonia was due to the effect on the hydrogen concentration of the different solubilities of the calcium sulphate and calcium nitrate, formed by reaction of the respective ammonium salts with calcium carbonate. According to his hypothesis the rate of ammonia lost by volatilisation should be: DAP > AS > AN.

In order to investigate this hypothesis a series of laboratory experiments was conducted with ammonium sulphate, ammonium nitrate and ammonium phosphates. In some of the experiments the ammonium salt was placed on the surface of the soil and in others it was mixed with the soil. Although the methods of placement might influence the absolute amount of volatilisation, this was considered to be of no significance as only the comparative volatilisation of ammonia is considered.

In 4 Fällen von Pleuraexsudat nichttuberkulöser Art fanden wir zwischen 2,3–5,3 % Eiweiß im Exsudat. In den 3 Fällen (Werte 3,45–4,89 und 2,30) war die febrile Periode ganz kurz, im 4. Falle (5,3) entstand ein Empyem.

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BESTEHEN BEZIEHUNGEN ZWISCHEN DER OBLIGAT URTICARIOGENEN UND DER FAKULTATIV EKZEMATOGENEN WIRKUNG DES ATROPINS?

Von

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W. Jadassohn und M. B. Sulzberger[1] stellten bei Untersuchungen über die Genese der Urtica fest, daß sowohl das vaguslähmende Atropin als auch das für den Parasympathicus indifferente Apoatropin bei intradermaler Einverleibung Quaddeln erzeugen kann, und zwar noch stärker als Atropin. Eine auffallende Analogie findet diese Tatsache in Versuchen, die der eine von uns (M.[2]) bei der Analyse der Atropinüberempfindlichkeit anstellte: Apoatropin erwies sich bei den drei geprüften Überempfindlichen als sogar stärker ekzematogen als das Atropin selbst. Von sonstigen Substanzen, die dem Atropin chemisch oder pharmakologisch nahestehen, „reizten" aber nur noch sehr wenige: Hyoscyamin, Homatropin und Scopolamin; viele andere, wie z. B. Benzoyltropein, Lactyltropein, Zimttropein sowie Tropin und Scopolin, Tropasäure und tropasaures Tropin waren wirkungslos.

Diese eigentümliche Kongruenz legt die Frage nahe, ob zwischen dem „obligat urticariellen" und dem „fakultativ ekzematogenen" Effekt dieser Substanzen Beziehungen bestehen — wobei wir als obligat urticariogen die in der Substanz begründete Fähigkeit verstehen, bei jedem Menschen Quaddeln zu erzeugen, als fakultativ ekzematogen die durch besondere „Disposition" des Individuums begründete ekzematogene Wirkung. Derartige Beziehungen wären aber von prinzipieller Wichtigkeit; wir haben es infolgedessen für notwendig gehalten, außer dem von W. Jadassohn und Sulzberger (l. c.) bereits untersuchten Apoatropin noch eine größere Anzahl anderer, näher oder entfernter verwandter Glieder der Atropingruppe zu prüfen, um zu entscheiden, ob diese Beziehungen in der Tat vorhanden sind. Es hat sich dabei herausgestellt, daß im Gegensatz zu der Atropinüberempfindlichkeit, bei welcher nur wenige Substanzen dieser Gruppe ekzematogen wirkten, die urticariogene Wirkung eine Eigenschaft aller geprüfter Gruppenangehöriger, vielleicht der ganzen Gruppe ist. Es bestehen also keine Beziehungen zwischen dem obligaten Quaddeleffekt und dem fakultativen Ekzemeffekt des Atropins und der anderen ekzematogen wirkenden Verwandten.

Beide Vorgänge können demnach unabhängig voneinander durch die gleiche Substanz ausgelöst werden: das ist der Fall beim Atropin, Apoatropin, Hyoscyamin, Homatropin, die sowohl obligat urticariogen als auch fakultativ ekzematogen wirken. Andere Substanzen hingegen lösen nur einen Vorgang aus, das ist der Fall bei den Tropeinen, den Spaltlingen des Atropins und des Scopolamin u. a. m. Die vermutete Kongruenz ist also nur eine scheinbare.

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TIEREXPERIMENTELLE STUDIEN ÜBER DIE HÄMOREGENERATORISCHE WIRKUNG DER SÄUREN.

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Von

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Durch meine, im Laufe der letzten Jahre bekanntgemachten Untersuchungen[1,2,3,4] scheint die Annahme berechtigt zu sein, daß die Vermehrung der sauren Bestandteile des Blutes, gleichviel ob diese Vermehrung in physiologischer, in pathologischer Weise oder experimentell zustande gekommen ist, die Zahl der roten Blutkörperchen im strömenden Blute vermehrt, indem sie einen formativen Reiz auf das erythropoetische Knochenmark ausübt.

Als Ergänzung zu den in meinen vorhergehenden Mitteilungen bekanntgemachten Literaturangaben möchte ich noch das vor mehr als 40 Jahren erschienene Buch Bunges (Lehrbuch der physiologischen und pathologischen Chemie, Leipzig 1887) erwähnen. Auf Grund von Zanders Untersuchungen gibt er zum ersten Male der Anschauung Ausdruck, daß „die Salzsäure gegen die Chlorose ein noch wirksameres Mittel sei, als das Eisen". Bunges theoretische Erklärungen dürften heute kaum mehr bestehen, es ist aber unzweifelhaft, daß er der erste war, der die auf die Hämopoese bzw. Hämoregeneration ausgeübte Wirkung der Säurenverabreichung erkannte.

Falls der Acidose tatsächlich die Bedeutung eines Knochenmarkreizes zukommt, liegt die Annahme auf der Hand, daß die Intensität der nach akuten Blutverlusten auftretenden spontanen Blutregeneration auch durch eine künstliche Acidose gesteigert werden kann. Um diese Annahme zu rechtfertigen, unternahmen wir Untersuchungen, deren Ergebnisse im folgenden bekanntgegeben werden.

Zu unseren Versuchen wählten wir gut entwickelte, gesunde Kaninchen. Die Grundlage der Untersuchungen war folgende: Sämtliche Kaninchen wurden durch Aderlaß in gleichem Grade anämisiert. Bei der einen Gruppe der anämisierten Kaninchen wurde die Intensität der spontanen Blutregeneration beobachtet, bei der anderen Gruppe wurde die Restitution nach Erzeugung einer künstlichen Acidose zum Gegenstand unserer Untersuchungen gemacht.

Die Zahl der untersuchten Versuchstiere war 12. Bei 5 (1. Gruppe) wurde die spontane, bei 7 (2. Gruppe) die nach Säureverabreichung beobachtete Regeneration untersucht. Die Blutentnahme wurde bei den Tieren beider Gruppen an der Vena jugularis ausgeführt. Der künstliche Blutverlust betrug bei sämtlichen Tieren 30 ccm pro Kilogramm Körpergewicht. Die Säure wurde täglich einmal peroral mittels Sonde verabreicht. Von den Tieren der 2. Gruppe erhielten 3 Salzsäure, 2 Phosphorsäure, 2 Schwefelsäure. Durch die Variierung der Säure konnten wir die evtl. Wirkung der Anionen ausschalten. Die Menge der täglich verabreichten Säure entsprach 2 ccm einer konzentrierten Lösung pro Kilogramm Körpergewicht. Die Säurebehandlung wurde so lange fortgesetzt, bis die ursprüngliche Zahl der roten Blutkörperchen erreicht war. An den der Blutentnahme vorangehenden Tagen wurde bei jedem Tier zweimal eine hämatologische Kontrolle unternommen, um die spontanen Schwankungen in Betracht ziehen zu können. Die hämatologische Kontrolle wurde im Anfang täglich, später zweitägig ausgeführt und das Blutbild auch noch weiterhin, nach vollständiger Restitution, beobachtet. Die hämatologische Kontrolle bestand in jedem Falle aus der Bestimmung der Zahl der roten und der weißen Blutkörperchen, des Hämoglobingehaltes, des qualitativen Bildes der roten und weißen Blutkörperchen und aus der Bestimmung der Serumrefraktion.

Da die Art und Weise der spontanen Regeneration nach Blutverlusten mit Hilfe klinischer Beobachtungen und von Tierexperimenten schon lange als geklärt aufgefaßt werden kann und sie sogar ein in Einzelheiten bekannter Vorgang ist, halten wir es für überflüssig, uns auch mit unseren diesbezüglichen

Beobachtungen zu befassen. Im weiteren beschränken wir uns nur auf Vergleiche; der Kürze halber teilen wir die in der Qualität und Intensität der Regeneration der Tiere beider Gruppen beobachteten Unterschiede zusammengefaßt mit. Von der Mitteilung der Versuchsprotokolle und eingehender numerischer Daten sehen wir ab.

Durch Aderlaß gelang es uns, die Erythrocytenzahl der Versuchstiere um 31—43 % zu vermindern. Die Verminderung des Hämoglobingehaltes wurde — die Versuchsfehler in Anbetracht ziehend — obigen Daten entsprechend gefunden. Dieser unveränderte Färbeindex konnte aber nur in den ersten Tagen nach der Anämisierung beobachtet werden, mit dem Eintritt der auffallenderen Regeneration überschritt die Regeneration der roten Blutkörperchen die des Hämoglobins. Diese Erscheinung schien bei den Tieren der zweiten Gruppe, d. h. bei den mit Säure behandelten Kaninchen gewaltiger ausgeprägt zu sein, als bei den Kontrolltieren. Nach BRUGSCH u. a. ist dieser verminderte Färbeindex der Ausdruck der gesteigerten Hyperaktivität des Knochenmarkes, indem die durch das übertrieben in Anspruch genommene Knochenmark erwirkte Hämoglobinsynthese hinter der Erythropoese zurückbleibt, infolgedessen an Hämoglobin ärmere rote Blutkörperchen in die Zirkulation gelangen. Hinsichtlich dieser Tatsache sollte unsere eben erwähnte Beobachtung berücksichtigt werden, daß nämlich die Verminderung des Färbeindexes bei den mit Säure behandelten Tieren stärker ausgeprägt war.

Bei beiden Gruppen angehörenden Versuchstieren hatten wir Gelegenheit, die als Hyperkompensation bekannte Erscheinung zu beobachten, bei welcher die Vermehrung der roten Blutkörperchen nicht beim ursprünglichen — vor der Blutentnahme beobachteten — Niveau anhielt, sondern dieses noch überschritt.

Die Hyperkompensation konnte aber bei keinem einzigen Fall dauernd beobachtet werden, die Erythrocytenzahl verminderte sich in einigen Tagen wieder bis zum vor der Venaesektion beobachteten Ausgangsniveau und stabilisierte sich dort alsbald endgültig.

Betreffs des Hämoglobins wurde diese Hyperkompensation entweder überhaupt nicht, oder höchstens in geringerem Maße beobachtet, so daß der Färbeindex seinen Ausgangswert eben beim Verschwinden der Hyperkompensationswerte der Erythrocytenzahl erreichte. Bei den mit Säure behandelten Tieren zeigte sich eine auffallendere Hyperkompensation und eine langsamere Abnahme derselben.

Obige Erscheinung spricht dafür, daß die — es sei mir der Ausdruck erlaubt — kinetische Energie der Regeneration der mit Säure behandelten Tiere sich als größer erwies als die der Kontrolltiere.

Die Regenerationsgeschwindigkeit der roten Blutkörperchen wird durch nachstehende Tabelle veranschaulicht.

	Qualität der Säure	Zeit der Regeneration	
1. Kontrolltiere (spontane Regeneration).			
Versuchstier Nr. 1	—	22 Tage	*Durchschnitt: 27 Tage*
„ „ 2	—	25 „	
„ „ 3	—	28 „	
„ „ 4	—	28 „	
„ „ 5	—	32 „	
2. Mit Säure behandelte Tiere.			
Versuchstier Nr. 6	Salzsäure	11 Tage	*Durchschnitt: 16 Tage*
„ „ 7	Schwefelsäure	15 „	
„ „ 8	Salzsäure	15 „	
„ „ 9	Salzsäure	16 „	
„ „ 10	Phosphorsäure	17 „	
„ „ 11	Phosphorsäure	18 „	
„ „ 12	Schwefelsäure	19 „	

Die in der Tabelle enthaltenen Angaben legen klar, daß die Regeneration durch die Säurenverabreichung erheblich gesteigert wird. Daß die Regeneration tatsächlich gesteigert ist, wird auch — neben obigen Angaben — durch diejenigen Beobachtungen bewiesen, die wir im Verlaufe der Untersuchungen des qualitativen roten Blutbildes machten. Nach diesen Beobachtungen waren die heterochromatischen Erythrocyten — deren Anzahl sich bekannterweise mit der Intensität der Erythrocytenregeneration proportionell erweist — im Blute der mit Säure behandelten Tiere in viel größerem Ausmaß vorzufinden als in dem der Kontrolltiere.

Kernhaltige Erythrocyten wurden während der Regeneration unter den der ersten Gruppe angehörenden Tieren bei 1 (20 %), bei den der zweiten Gruppe angehörenden bei 3 (43 %) gefunden.

Die Untersuchung der Serumrefraktion ergab bei den zwei Gruppen der Versuchstiere keinen prinzipiellen Unterschied, wenigstens keinen so hochgradigen, welcher die, in der Regenerationsgeschwindigkeit beobachtete Differenz auch nur teilweise erklären dürfte.

Was die weißen Blutkörperchen anbelangt, hatten wir in der ersten Phase der Regeneration bei beiden Gruppen Gelegenheit, die als „posthämorrhagische Leukocytose" bekannte Erscheinung zu beobachten. Diese posthämorrhagische Leukocytose zeigte sich bei den mit Säure behandelten Tieren viel erheblicher als bei den Kontrolltieren. Besonders auffallend war die hochgradige Vermehrung der myelogenen Leukocytenelemente, der Granulocyten, weiterhin die Tatsache, daß die regeneratorische „Linksverschiebung" bei den mit Säure behandelten Tieren mehr ausgesprochen war als bei den der ersten Gruppe angehörenden.

Diese Erscheinung berechtigt zur Annahme, daß nicht nur der erythropoetische, sondern auch der leukopoetische Teil des Knochenmarkes durch die Acidose beeinflußt wird. Diese Annahme wird teils durch meine eigenen obenerwähnten Untersuchungen betreffs des Blutbildes bei der diabetischen Acidose, teils auch durch diejenigen Beobachtungen, nach denen mit Säureverabreichung eine neutrophile Leukocytose im Blute der Versuchstiere erzeugt werden kann, gerechtfertigt.

Nach unseren Versuchen vermag demnach die künstliche Acidose auch das sich in regeneratorischer Hyperfunktion befindende Knochenmark zu stimulieren, d. h. daß *die nach Blutverlust erfolgende Regeneration durch Säureverabreichung gesteigert werden kann.* Unserer Meinung nach bietet diese Beobachtung Möglichkeiten, auf Grund deren gewisse therapeutische Versuche vorgenommen werden dürften.

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KURZE WISSENSCHAFTLICHE MITTEILUNGEN.

BEOBACHTUNGEN DES CAPILLARKREISLAUFES UND LEBENDER PARASITEN IM STRÖMENDEN BLUTE.

Von

FRITZ A. LEHMANN.

Die Arbeit VONWILLERS: „Die Beobachtung des Blutes am lebenden Menschen*", veranlaßt mich schon heute, Mitteilung über direkte Beobachtung des Capillarkreislaufes am Warmblüter und von im Blute kreisenden Parasiten zu machen, Untersuchungen, die sich in wesentlichen Punkten mit denen VONWILLERS berühren.

Als Versuchsobjekt erwies sich mir das Ohr der weißen Maus, besonders der jungen, als sehr geeignet, da es erlaubt, bei *durchfallendem* Licht mit Ölimmersion bei *stärkster Vergrößerung* zu beobachten. Das Objekt ist infolge seiner außerordentlichen *Einfachheit* zur Demonstration des Kreislaufs am Warmblüter ebenso zu gebrauchen wie bisher die Froschzunge am Kaltblüter.

* Klin. Wschr. 1929, Nr 18.

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THE DEVELOPMENT OF FIBROSIS IN THE KIDNEY.

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(PLATES XIV AND XV.)

RENAL fibrosis is generally taken to be the result of proliferative activity on the part of the interstitial cells of the kidney, it being assumed that the collagenous fibres are a product of connective tissue cells. I have recently had an opportunity of studying this question in relation to an experimental nephritis in rats in which fibrosis is a prominent feature, and my findings do not seem to bear out this assumption. On the contrary they lead me to favour a view which has been gaining ground in recent years, namely that connective tissue is of non-cellular origin. The observations which I wish to put forward are largely a matter of histological interpretation and as such may not be regarded as sound foundations for such a view. Nevertheless, since the cellular assumption is itself based on histological interpretation, it is open to criticism on histological grounds, and it is on this account that I feel justified in recording my observations in the rat nephritis. Lesions of the kind have been described before, but discussions on the interstitial changes have been mainly concerned with the problem of their relationship to the parenchymatous degeneration (see Dickson, 1912; Aschoff, 1912; Suzuki (quoted by Aschoff); and Oliver, 1915; on uranium nephritis) and not with the mechanism of connective tissue formation. In this respect the rat nephritis which I wish to discuss opens a field not hitherto fully explored.

The nephritic lesion.

This has already been described (Gough, Duguid and Davies, 1933; Duguid, 1933-34).

Rats are maintained for periods of 20-30 days on a mixture of bread and potatoes to which has been added acid sodium phosphate, 5 parts per 100, while at the same time daily doses of 10,000-40,000 units of calciferol are given by mouth. At the end of the period of treatment the animals are placed on a normal mixed diet and allowed to recover. The majority develop a renal lesion which becomes chronic and progressive. It consists (NaH₂PO₄)

Phos

of a parenchymatous degeneration in which groups of tubules atrophy and disappear, leaving scar-like areas of fibrosis in their place. Other tubules become distended with albuminous coagulum so that the kidney as a whole is distorted with its surface pitted and granular (fig. 1). In the more severe cases secondary changes such as hyaline degeneration of the glomeruli and arterioles may appear, and many glomeruli may be totally destroyed and obliterated. Even the arteries may appear thickened and tortuous, so that practically all the histological features of a human chronic nephritis may be reproduced. The condition offers an excellent opportunity for the study of fibrous tissue formation since it entails a replacement fibrosis, which can be followed through its different stages of development by killing off the animals at different periods.

The material upon which this study is based comprised more than 300 rats taken at various stages of the disease from the sixth to the six hundredth day, and showing various degrees of renal destruction.

Observations on the fibrotic process.

The development of the fibrosis is most easily followed in the renal cortex because there the interstitial tissue normally consists of little more than a delicate framework of reticulum, occupying a minimum space between the tubules. Collagenous material is seldom seen except in relation to the glomeruli and blood vessels, and when any appears amongst the tubules it may be regarded as pathological. In the medulla the interstitial tissue is normally more plentiful and mixed in character, consisting of both collagen and reticulum, so that to distinguish new-formed collagen from the original interstitial tissue in this region is less easy. For the study of interstitial tissue in the kidney I have found the Bielschowsky-Foot technique, using van Gieson as a counterstain (Foot and Ménard, 1927), the most valuable.

In the rat nephritis interstitial fibrosis arises by two different processes, one consisting of a condensation and transformation of the original reticular framework of the kidney, the other of an actual new formation of tissue. The first process may be described in a few words. When tubules atrophy and disappear the network of reticulum which encloses them collapses so as to form dense masses of fibrils in which endothelial and other cells, including degenerate remnants of epithelium, may be crowded together (figs. 2 and 3). In sections stained with hæmalum and eosin, such masses have the appearance of fibrous scars, but with special connective tissue stains they are usually shown to contain no actual new formation of tissue, merely a change in the arrangement and distribution of the old. It is only in the later stages of this process that collagen appears, and then it seems to arise simply by a transformation of the reticulum fibres. In the early stages these fibres remain distinct and discrete, but later they lose their

FIG. 1.—Kidney of rat which received NaH_2PO_4 and 40,000 units of calciferol for 23 days and subsequently lived 130 days. The tubules are practically all affected, some dilated, others shrunken and replaced by fibrous tissue. Hæmalum and eosin. ×30.

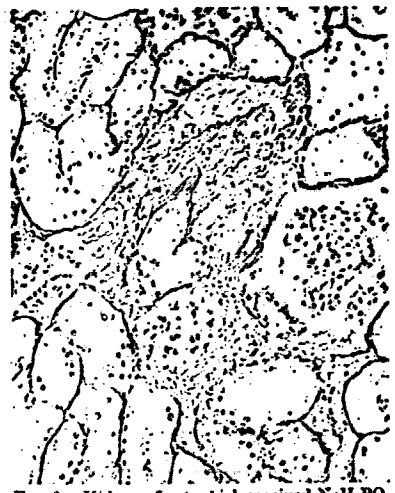

FIG. 2.—Kidney of rat which received NaH_2PO_4 and 20,000 units of calciferol and died on 19th day of treatment. A small area of tubular atrophy is shown in which the reticular framework of the cortex has fallen together so as to form a dense mass of fibrils. Bielschowsky-Foot method. ×185.

FIG. 3.—Kidney of rat which received NaH_2PO_4 and 20,000 units of calciferol for 28 days and subsequently lived 325 days. A much more severe degree of atrophy occurs in which the reticular framework forms a network of dense shrunken fibrils with remnants of tubules in its meshes. Bielschowsky-Foot method. ×200.

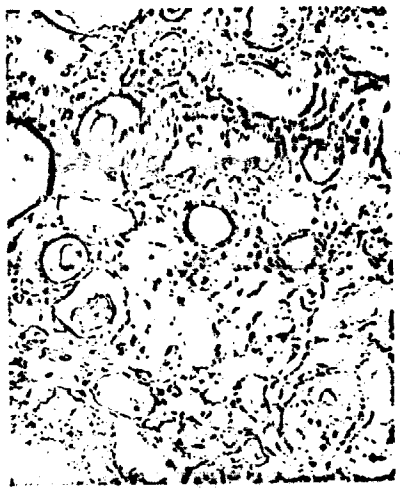

FIG. 4.—Kidney of rat which received NaH_2PO_4 and 40,000 units of calciferol for 25 days and subsequently lived 82 days. There is intense tubular degeneration and the tissue appears to be infiltrated with hyaline material. Hæmalum and eosin. ×200.

sharp outlines while their silver staining properties fade. At first they stain brown, but later they fail entirely to take up silver and assume the pink stain with van Giesson, so that they have all the appearance of collagen fibres. As the nephritis advances the cells tend to diminish and disappear, the tissue shrinks and becomes denser and in the end all that may remain is a thin streak of fibrous tissue representing what was originally a wide area of parenchyma. In the milder cases this simple process of condensation and de-differentiation of the reticulum accounts for most of the picture.

The second process is best seen in the more severe lesions. It is of a more complex character and is usually superimposed on the change already described. It involves a true increase of interstitial tissue, and it appears to depend in the first place on an influx of new ground substance into the tissue spaces. The origin and nature of this substance is not clear, but both its appearance and distribution suggest a relationship to œdema. In the early stages of the nephritis, especially those seen during the period of treatment, œdema is a frequent feature, chiefly in the neighbourhood of the degenerate tubules. The interstitial spaces appear swollen with fluid and the reticulum fibres split up into finer strands. As a rule the condition has all the appearances of a simple transudation, but sometimes a coagulum seems to form which is slightly refractile and which, in sections impregnated with silver, may show a faint granularity. In the later stages of the nephritis, as seen in rats killed some time after the treatment has been stopped, the œdema may have disappeared, leaving the tissues in a fairly compact state, but in the more severe lesions there sometimes remains, in place of the œdema, a hyaline ground substance (figs. 4 and 5), and it is this which seems to form the basis of connective tissue growth. The appearance and staining reactions of this substance are variable. As a rule it is homogeneous and translucent, but sometimes in places it appears more dense and opaque. With van Gieson it shows colours ranging from pale yellow to deep pink, and it is this feature which seems to proclaim its relationship to connective tissue formation. Sometimes the yellow colour can be seen gradually merging into the pink, especially around the basement membranes of the tubules and on the surface of the reticulum fibres, as if the ground substance were condensing on these to form thickenings of the fibres. In the more advanced cases it may stain an almost uniform deep pink. With other connective tissue stains a similar impression is produced. Mallory's phosphomolybdic acid-aniline blue colours this ground substance a variable depth of blue, the deeper shades being seen especially around the basement membranes of the tubules, while eosin produces that familiar appearance which has been described as hyaline thickening of the basement membrane. Thus there seems every reason to conclude that this hyaline ground

substance is itself changed into collagen and actually forms the new connective tissue.

In the presence of this change, the reticulum fibres may at first remain distinct, although they are usually separated into finer strands by infiltration of the hyaline ground substance into their interstices. Later, when the ground substance has become condensed and pink-staining, they tend to lose their argyrophil character so that they also take the pink of the van Gieson and are no longer clearly distinguishable from the rest of the interstitial substance (fig. 6), except for the somewhat fibrillated appearance which they sometimes impart to it. Thus in the end both the original reticulum and the new-formed ground substance combine to form pink-staining masses of collagen.

The relationship of this process to the interstitial cells is the point on which my attention has been especially focussed. In the early stages of the nephritis there is sometimes, along with the œdema, an infiltration of round cells into the tissue spaces as well as an increase of endothelial and other cells, and the question whether any of these are responsible for the new formation of connective tissue is the main issue. The answer can as yet only be offered as a matter of opinion, but there would seem to be little reason to regard the two as essentially related. Although the cellular increase and the new formation of tissue may often coincide, their relative proportions are by no means constant and in fact the interstitial substance is often found in greatest quantities when there are few cells (fig. 5). It is true that the cells and fibrils are often arranged in a rough alignment such as might suggest that they are growing in conjunction, and Wolbach (1933) has cited this common feature as evidence in favour of the cellular hypothesis, but the arrangement is in my opinion of little significance. The fibril formation is due in the first place to the original reticulum, which, although it becomes dedifferentiated so far as staining is concerned, retains its fibrillary form, and the cells which lie amongst it will naturally adjust themselves to this by arranging themselves in alignment with the fibrils. The hyaline ground substance which seems to form the basis of collagen is a homogeneous material apparently diffused through the tissues and more like a fluid infiltration than a formed product of any particular cell or group of cells. Its nature is unknown, but everything in its appearance seems to suggest an inflammatory or degenerate product of an exudative character.

Further light on fibrous tissue formation is sometimes afforded by the glomerular degeneration which appears in the more severe cases. Many of the glomeruli are totally destroyed and their ultimate state is fibrosis. They first undergo a kind of hyaline degeneration, their capillary walls becoming swollen and fused

FIG. 5.—Kidney of rat which received NaH_2PO_4 and 40,000 units of calciferol for 25 days and subsequently lived 161 days. The interstitial spaces are swollen with hyaline material. Hæmalum and eosin. ×200.

FIG. 6.—Same case as in fig. 3, showing an area of dense fibrosis, probably representing a destroyed glomerulus. The reticular fibres at the margins of the area are stained black with silver, but in the area itself they are no longer differentiated. ×200.

FIG. 7.—Same case as in fig. 4, showing hyaline degeneration of a glomerulus. ×200.

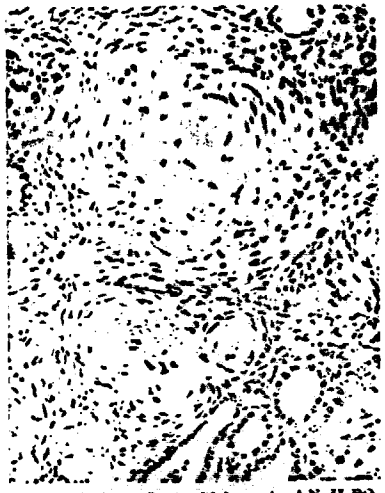

FIG. 8.—Kidney of rat which received NaH_2PO_4 and 40,000 units of calciferol for 25 days and subsequently lived 59 days, showing a glomerulus and its arterioles completely fibrosed. Hæmalum and eosin. ×200.

and their capsular spaces obliterate. Later, their cells fade and disappear until nothing is left but masses of hyaline material (fig. 7). At first these stain yellow with van Gieson but later they take a pink colour, so that they have the appearance of collagen. For a time these remnants of glomeruli remain clearly distinguishable as dense fibrous bodies (fig. 8) having a somewhat convoluted internal structure, but later they shrink and become more compact while their outlines become less distinct, until finally they merge with the surrounding connective tissue and are lost. In this process there is no sign of any removal of tissue or substitution, simply a dedifferentiation and transformation into collagen.

A similar process may sometimes be seen in degenerate arterioles and even in tubules. When the tubules atrophy they collapse and form solid cords of cells which gradually disappear. There is nothing to suggest that their disappearance involves phagocytosis or absorption. The cells merely lose their nuclear staining and, becoming homogeneous, take first a yellow and later a pink colour with van Gieson. Ultimately they become uniformly pink so that they are no longer distinguishable from the surrounding interstitial tissue, and thus it would seem as if the cytoplasm were itself actually changed into collagen. Apparently therefore degenerate tissue of almost any kind, including destroyed glomeruli and atrophic epithelium, may provide the material from which fibrous tissue is formed.

Discussion.

Since the middle of last century the current views on connective tissue formation have been strongly influenced by the cellular hypothesis. Some authorities have held that the fibres are formed in the cell protoplasm, others that they are formed from the fluid intercellular substance which is itself a secretion of the cell, but nearly all have agreed in regarding the cell as the essential tissue builder. It is only recently that the idea of connective tissue arising from a substance of non-cellular origin has been to any extent entertained.

In 1910 Hertzler concluded from a study of organisation in the peritoneum that fibrous tissue formation was comparable with fibrin formation in blood coagulation, the cell taking no active part in the process. Later Baitsell (1916) described the transformation of fibrin into connective tissue in wound organisation at regions where no cells were present, and he also (1915) described fibril formation in the plasma medium in tissue cultures. Alfejew (1925-26) and Harrison (1924-25) considered that fibres, in embryonic development, arose from an amorphous ground substance without any direct connection with cells, and Foot and Day (1925) suggested that fibro formation in tumours was something in the nature of a precipitation or accretion in the intercellular ground substance. This has been largely corroborated by Maximow (1928) from his observations on tissue culture. In a study of nerve regeneration Nagcotte (1922) concluded that various materials including dead and degenerate tissues and cells may go to the formation of

collagenous connective tissue, and recently Day (1936) has ventured the suggestion that the œdema fluid may provide the natural substrate for the fibrous tissue formation which occurs in chronic lymphatic obstruction. Further support has been lent to the non-cellular theory by the work on tissue culture of Doljanski and Roulet (1933) and von Jeney and Töröö (1936-37). These writers have described fibres developing in the culture medium at parts distant from the living cells and have concluded that their formation is a colloidal chemical reaction rather than a cytological one. The latter authors believe that a specific substance, probably identical with ascorbic acid, is the essential agent in this reaction.

There is thus a considerable body of opinion favouring the non-cellular theory of connective tissue formation; nevertheless, in so far as the pathology of renal disease is concerned, the cellular hypothesis still prevails, and most authorities regard interstitial fibrosis as a proliferative phenomenon.

While it is admitted that histology may mislead there are some points of evidence in the rat nephritis which it would seem fairly safe to accept. There is evidence for example that fibrous tissue is formed by a transformation of the original reticulum. It is generally acknowledged even by observers who adhere to the cellular hypothesis (*e.g.* Mallory and Parker, 1927) that such a transformation may occur, and there are in fact many authorities who regard reticulum as the natural precursor of collagen. This alone is strong evidence against the cellular hypothesis because, as has been pointed out, the fibrosis in milder cases of the rat nephritis may be nothing more than a condensation and de-differentiation of the original reticulum. In the more severe cases the obliteration of glomeruli is a simple fibrous transformation in which the cells seem to play an entirely passive role. Thus, leaving all other points out of consideration, there is evidence enough that renal fibrosis may occur independently of cells. There is no doubt that other processes may contribute, and I have described above a hyaline ground substance which I regard as the basis of much of the new formation of collagen, but as yet little is known of the nature of this ground substance and the question of its importance in tissue formation is left for further investigation. The important point in the rat nephritis is the total absence of evidence that connective tissue arises from any particular cell, or that it depends on the proliferative activity of any cells. All the evidence on the contrary seems to point to the fibrosis as a degenerative or retrogressive phenomenon rather than a proliferative one.

In describing this experimental lesion in rats I have used the term "nephritis" because I regard the changes as differing in no important essential from those seen in certain types of the human disease bearing that name. The two lesions correspond histologically and there seems no reason to suppose that they differ in their mode of development. In the rat nephritis fibrosis appears to be the

natural process whereby the kidney disposes of tissues which are no longer serving their natural purpose, and in view of this it would seem that the prevailing conception of interstitial nephritis as a reactive cellular change requires to be revised.

Conclusions.

The development of pathological fibrosis has been studied in an experimental nephritis in rats produced by the oral administration of acid sodium phosphate and calciferol.

No evidence has been found to support the cellular theory of connective tissue formation or to suggest that interstitial cell proliferation is an essential factor in renal fibrosis.

Renal fibrosis seems to be produced mainly by the collapse and condensation of the original interstitial framework of the kidney around atrophic tubules with subsequent transformation of the reticulum and other tissues into collagen.

In some cases an infiltration of a hyaline ground substance into the tissue spaces is seen and this also appears to form the basis of new connective tissue formation.

All the evidence seems to point to renal fibrosis as a degenerative or retrogressive rather than a proliferative phenomenon.

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Zur Frage der Resorption von Polyphosphaten *

Von

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(Eingegangen am 15. April 1965)

Alle, wie kaum für einen anderen Lebensmittelzusatzstoff durchgeführten oralen chronischen Toxitätsstudien brachten für die Gruppe der Polyphosphate (= linearkondensierte Phosphate mit einem Kondensationsgrad von 2 und höher) ein mit dem Monophosphat (= Orthophosphat) pharmakologisch-toxikologisch einheitliches Bild. Polyphosphate unterscheiden sich nämlich bei oraler Zufuhr weder untereinander noch von der Wirkung von Monophosphat und sind daher physiologisch qualitativ und quantitativ auf jeder Konzentrationsstufe gleich zu bewerten (HAHN, LANG).

Dieses Ergebnis läßt sich sowohl durch den chemischen Abbau (THILO) als auch die bekannte enzymatische Aufspaltung der Polyphosphate (MATTENHEIMER) erklären, zumal die bisherigen stoffwechselchemischen Untersuchungen einheitlich feststellten, daß lediglich Monophosphat und daneben vielleicht kleinere Mengen Diphosphat (= Pyrophosphat) bzw. Oligophosphate (= papierchromatographisch trennbare Einzelglieder der polymerhomologen Reihe der Polyphosphate mit einem Kondensationsgrad $n \leq 12$) zur Resorption gelangen (VAN GENDEREN et al., SCHREIER und NÖLLER, LANG u. Mitarb.).

Trotzdem wurde über eine peroral kardiale toxische Polyphosphatwirkung berichtet (EICHHOLTZ u. Mitarb.), was aber schon durch HAHN als experimenteller Irrtum widerlegt wurde. Solche Polyphosphatwirkungen sind aber dann grundsätzlich auszuschließen, wenn der endgültige und sichere Beweis dafür erbracht ist, daß überhaupt nur Monophosphat, also nicht auch Oligophosphate, resorbiert werden.

Durch kombinierte Anwendung der derzeit optimalen Methoden zur Kontrolle von Stoffwechselvorgängen, nämlich ^{32}P-markierte Polyphosphate und Papierchromatographie, klärten wir die Frage nach der Art der Resorption von Polyphosphaten. Sprague-Dawley-Ratten wurden jeweils 5 ml einer bestimmten Lösung von ^{32}P-markiertem Tetranatriumdiphosphat ($Na_4P_2O_7$), Pentanatriumtriphosphat ($Na_5P_3O_{10}$) und Natriumpolyphosphat mit 64% P_2O_5 mit der Schlundsonde appliziert. Die Verweildauer dieser Polyphosphate im Magen-Darm-Trakt wurde auf 1, 2, 4, 8 und 16 Std festgelegt und danach die Ratten decapitiert und das erhaltene Frischblut in zwei Anteilen untersucht. Der eine Teil diente zur Bestimmung der ^{32}P-Aktivität im Blut als Funktion der Zeit und der Art des jeweils verabreichten Polyphosphats und der andere zur Identifizierung des im Blut vorhandenen Phosphats und der daran gebundenen ^{32}P-Aktivität.

Unsere Untersuchungen ergaben nun, daß das mittels Trichloressigsäure enteiweißte Vollblut der Versuchstiere im Chromatogramm nur zwei phosphathaltige Flecken zeigte, die mittels Vergleichschromatogrammen, Rf- und Pk-Werten und dem gleich behandelten Blut einer Kontrollratte als Monophosphat und Adenosinmonophosphorsäure (= AMP) identifiziert werden konnten. Die ^{32}P-Aktivität wanderte dabei in Abhängigkeit von der Verweildauer im Magen-Darm-Trakt bzw. der durch den Resorptions- bzw. Phosphorylierungsvorgang bedingten zeitlichen Verschiebung vom Monophosphat in die AMP, d.h. das ausschließlich aufgenommene anorganische Monophosphat wird in die organische Bindung der AMP übergeführt und so dem Intermediärstoffwechsel zugeleitet. Die bisher geäußerten Zweifel an der Resorption von Polyphosphaten (einschl. Oligophosphaten) waren also berechtigt, denn die Zellmembranen sind für kondensierte Phosphate nicht durchlässig und die Adenosinphosphorsäureester des Serums bzw. die bisher an Stoffwechselkäfigen nicht mögliche einwandfreie Trennung von Harn und Kot der Versuchstiere täuschten eine Resorption von Oligo- und damit niedermolekularen Polyphosphaten vor.

Die biologischen Beurteilungen der Polyphosphate durch die FAO/WHO und die Deutsche Forschungsgemeinschaft fanden hierdurch eine weitere experimentelle Bestätigung, denn oral zugeführte Polyphosphate wirken nur in dem Umfange im Organismus, in dem sie zu ausschließlich resorbierbarem Monophosphat aufgespalten werden. Daher ist deren tägliche Zufuhr im Rahmen der täglichen Gesamtphosphatzufuhr (natürlicher P-Gehalt der Nahrung + zugesetzte Mono- und Polyphosphate) zu berücksichtigen.

Andere Vorstellungen über schädliche, insbesondere spezifische „Giftwirkungen" der Polyphosphate sind daher zurückzuweisen.

Zusammenfassung. Zur Klärung der Frage nach der Resorption von Polyphosphaten wurden Lösungen von ^{32}P-markiertem Diphosphat, Triphosphat und einem Natriumpolyphosphat mit 64% P_2O_5 Ratten mit der Schlundsonde appliziert. Nach einer Verweildauer von 1, 2, 4, 8 und 16 Std dieser Polyphosphate im Magen-Darmtrakt wurden die Ratten dekapitiert und das erhaltene Frischblut radiometrisch und papierchromatographisch untersucht.

Im enteiweißten Vollblut konnten nur Monophosphat und AMP nachgewiesen werden, d. h. es wird ausschließlich anorganisches Monophosphat resorbiert, das durch enzymatische Aufspaltung der Polyphosphate gebildet wird. Dieses Monophosphat wird dann in die organische Bindung der AMP übergeführt und so dem Intermediärstoffwechsel zugeleitet.

Polyphosphate besitzen also keine „spezifischen Giftwirkungen" und unterscheiden sich in der toxikologischen Wirkung weder untereinander noch vom Monophosphat, weshalb ihre Beurteilung mit der von Monophosphat gleichgesetzt wurde.

Die tägliche Zufuhr der Polyphosphate mit der Nahrung ist daher nur im Rahmen der täglichen Gesamtphosphatzufuhr (natürlicher P-Gehalt der Nahrung + zugesetzte Mono- und Polyphosphate) zu berücksichtigen.

Summary. Solutions of P^{32} radioactively-labelled tetrasodium diphosphate, sodium triphosphate, and sodium polyphosphate with a 64% P_2O_5 content were administered to rats by a stomach tube for the purpose of absorption evaluation of polyphosphates. After 1, 2, 4, 8 resp. 16 hour-remaining of the polyphosphates in the intestinal tract the rats were decapitated and the fresh blood thus obtained was examined by radiometrical and paperchromatographical method.

In the deproteinized blood, only monophosphate and AMP could be identified, i. e. exclusively inorganic monophosphate formed by enzymatic degradation of the polyphosphates is absorbed. This monophosphate is then converted into the organic bond of AMP and thus prepared for entry into the intermediary metabolism.

Polyphosphates do therefore not produce any "specific toxicological effects", concerning their toxicological effec-

* Eine ausführliche Publikation wird in Kürze in der „Zeitschrift für Ernährungswissenschaft" erfolgen.

...ey do not distinguish from each other nor from monophosphate. Thus, for purposes of toxicological evaluation, polyphosphates are considered equivalent to monophosphate.

The daily intake of polyphosphates with nutrition is therefore, related only to the daily total phosphate intake (= total dietary phosphorus intake from both food and added mono- and polyphosphates).

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Plasma-Katecholamine bei Diabetes mellitus unter N_1,n-Butylbiguanid *

Von

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(Eingegangen am 23. April 1965)

Seit der Einführung der Biguanide in die Therapie des Diabetes mellitus[10, 14, 3] wurden immer wieder Beobachtungen mitgeteilt, die den Gedanken nahelegen, daß der therapeutische Erfolg insbesondere beim sog. „brittle Diabetes" („smoothing effect")[3] über eine unmittelbare Beeinflussung des Nebennierenmarks zustande kommen könnte. Mit unseren Untersuchungen über den Wirkungsmechanismus des N_1,n-Butylbiguanid (Buformin)[6, 10] war ein adrenerger Effekt, den als erster CLARK[2] für einfache Guanidinderivate und später FRANK u. Mitarb.[4] für das Synthalin als durch DHE hemmbar beschrieben sowie in jüngster Zeit KRONEBERG und STOEPEL[7] für Phenyläthylbiguanid fanden, auch für das Buformin nachgewiesen worden, jedoch liegen dieser Aussage bisher nur indirekte Schlüsse zugrunde; eine Messung der Katecholamine im Blut unter der Einwirkung von Biguaniden wurde nicht durchgeführt.

Um den scheinbaren Widerspruch zwischen dieser adrenergen (eine Hyperglykämie auslösenden) und einer fraglichen adrenolytischen (zu einem stabilisierenden Effekt auf die Kohlenhydratbilanz des „labilen Diabetikers" führenden) Wirkung der Biguanide aufzuklären, untersuchten wir unter verschiedenen experimentellen Bedingungen[9] den Einfluß des Buformins auf das Nebennierenmark und ferner auf die Glykoneolyse, um nach einem adrenalinähnlichen Effekt des Buformin per se zu suchen. Im folgenden soll zunächst das Verhalten der Gesamtkatecholamine und des Adrenalins im Plasma von diabetischen Patienten und von Normalpersonen dargestellt werden.

Methodik

50 unausgewählte, an einem manifesten Diabetes mellitus erkrankte Patienten unseres Institutes erhielten eine wäßrige Buforminlösung 4%** langsam intravenös injiziert; davon 24 eine Dosis von 6 mg, 24 eine solche von 8 mg Buformin pro kg Körpergewicht, das Injektionsvolumen betrug 1,4 bis 2,0 ml pro 10 kg. 12 weitere Patienten dienten als Kontrollpersonen und erhielten das gleiche Volumen physiologische Kochsalzlösung, darüber hinaus bekamen zwölf junge gesunde Normalpersonen 6 mg Buformin pro kg Körpergewicht injiziert. Die Blutentnahmen erfolgten jeweils vor (= Ausgangswert) sowie 60, 120 und 240 min nach der Injektion. Neben der Ermittlung der Plasma-Gesamtkatecholamine und des Adrenalins im heparinisierten Blut fluorimetrisch nach[1] (Doppelbestimmung)*** wurde die Bestimmung von Glucose, α-Aminostickstoff, freien Fettsäuren, Aceton, anorganischem Phosphat, und Kaliumionen vorgenommen; über Veränderungen des Blutspiegels dieser Substanzen soll jedoch gesondert berichtet werden[12].

Nach 240 min mußten die Versuche beendet werden, da den Probanden eine längere Versuchsdauer wegen Gefährdung der Stoffwechselbilanz und wegen der für die Analysen erforderlichen relativ großen Blutmengen nicht zugemutet werden konnte. Aus diesen Gründen erfolgte auch die Trennung der diabetischen Patienten in zwei „überlappende Kollektive" mit nur je drei Blutentnahmen zu den Zeiten 0, 60, 120 min und 0, 120, 240 min zu jeweils zwölf Patienten, die Werte 0 und 120 min sind dadurch mit je 24 Patienten besetzt.

Wegen der außerordentlich großen (aber leicht erklärbaren) biologischen Streubreite der Ausgangswerte mußten wir uns entschließen, den jeweiligen vor der Injektion ermittelten Ausgangswert eines Versuches bei einer Versuchsperson als 100% anzugeben und die zugehörigen Meßwerte nach der Injektion darauf zu beziehen. Die Prüfung der Unterschiede der einzelnen Abweichungen auf Signifikanz erfolgte mittels doppelter Varianzanalyse.

Herrn Diplom-Mathematiker E. JUTZI danken wir für die mathematische Bearbeitung, Frau R. DETZMANN für sorgfältige technische Assistenz.

Ergebnisse

Zunächst konnten wir feststellen, daß die parenterale Gabe von Buformin in unseren Versuchen bei keinem Patienten Unverträglichkeitserscheinungen toxischer Natur auslöste. Mit diesem Befund sind die noch immer bestehenden Unklarheiten[3] über die Genese der die Biguanidtherapie begleitenden gastro-intestinalen Beschwerden als beseitigt anzusehen: die Nebenwirkungen verdanken ihre Entstehung nicht zentralen, sondern ganz offenbar *lokalen* Reizen.

Bei allen diabetischen Patienten (n = 59) fanden wir vor der jeweiligen Injektion einen Plasmaspiegel von 18,86 ± 1,61 μg/l Gesamt-Katecholaminen und 9,34 ± 0,88 μg/l Adrenalin, bei den gesunden Normalpersonen (n = 12) von 20,25 ± 2,87 μg/l Gesamt-Katecholaminen und 11,24 ± 2,42 μg/l Adrenalin.

Bei den diabetischen Patienten kommt es nach Injektion von physiologischer Kochsalzlösung („Kontroll-Patienten") zu einem kontinuierlichen Abfall sowohl der Gesamt-Katecholamine als auch des Adrenalins bis auf 76 bzw. 70% des Ausgangswertes 240 min post injectionem. 6 mg Buformin pro kg Körpergewicht führen zu einem Abfall der Gesamt-Katecholamine bis zu 81% und des Adrenalins bis zu 56% im Maximum bei 120 min post injectionem, danach streben die Werte allmählich dem Ausgangswert zu. Der Unterschied des Adrenalinwertes bei 120 min vom zugehörigen NaCl-Wert ist bei $p < 5\%$ signifikant.

8 mg Buformin pro kg Körpergewicht bewirken 60 min post injectionem keine Veränderung der Gesamt-Katecholamine, erst 120 min post injectionem wird ein Abfall auf 75% erreicht, der bis 240 min nahezu anhält. Das Adrenalin steigt 60 min post injectionem auf 156% des Ausgangswertes an, um dann kontinuierlich bis auf 56% 240 min post injectionem abzufallen. Die individuelle Variation der Adrenalin-Reaktion 60 min post injectionem ist außerordentlich groß und daher die Abweichung vom Ausgangswert bei $p = 5\%$ nicht signifikant (Tabelle).

Aus den bereits erwähnten methodischen Gründen war eine weitere zeitliche Verfolgung des Adrenalin-Blutspiegels leider nicht möglich. Die untersuchten Normalpersonen, bei denen wir uns aus äußeren Gründen auf lediglich eine Belastung mit 6 mg Buformin pro kg Körpergewicht beschränken mußten, zeigen keine Veränderungen der beiden Meßgrößen während der Versuchsdauer (Tabelle).

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* Über einen Teil der Ergebnisse wurde erstmalig berichtet auf der Tagung der Internisten Mecklenburgs vom 3./4. 7. 64 in Greifswald.

** Wir danken dem Arzneimittelwerk Dresden, insbesondere dem Leiter der Forschungsabteilung, Herrn Dr. CARRAUS, für die großzügige Bereitstellung von Versuchsmengen dieses Präparates und der Oberapothekerin an der Universitätsapotheke Greifswald, Frau Dr. WOLLMANN, für die sachevolle Zubereitung einer für diese Injektion geeigneten Buforminlösung.

*** Für die Berechnung der Werte wurde die Extinktion eines inneren Adrenalin-Standards zugrunde gelegt (l-Adrenalin-bitartrat, Chemische Werke Grünau).

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ROLE OF ADSORPTION IN THE DEPOSITION OF CHOLESTEROL IN ATHEROSCLEROSIS

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ANDREW GEMANT, PH.D., M.D.*

In a previous paper[1] the preparation and properties of in vitro cleared cholesterol solutions were described. The present paper deals with the deposition by adsorption of this cleared cholesterol on solids chemically related to the intima of arteries, as well as with the various factors that promote or inhibit this adsorption.

As stated in the previous paper, adsorption is only one of the many facets of the complicated mechanism of atheroma formation, the major part of this mechanism being metabolic: enzyme and hormone controlled. Patients with coronary artery disease show abnormal lipid metabolism, a tendency reversible by administration of heparin[2].

There are, on the other hand, observations that point to the role of physicochemical processes, such as adsorption, in atherosclerosis. Cholesterol found in atheromas is not locally synthetized, but originates from the blood. The intima of large arteries is lacking in capillaries and lymphatics, except those developed secondarily in atheromas. Blood in the wall is supplied by diffusion from the lumen. As a result, large particles in the plasma are retained by a filtration type process and tend to accumulate.[3] As for solutes of smaller size, they will be distributed between the liquid phase and solid surfaces by means of adsorption forces. This role of adsorption is seen also from the fact that the deposition of lipids is often not the primary, rather a secondary process[4] in which the chemical composition of the vessel wall is an important factor. An analogous role of adsorption occurs in the kidneys: in amyloid nephrosis combined with hypercholesterolemia the concentration of cholesterol in the urine is increased, leading to its deposition in the renal tubular epithelium.[5]

In the present work three main factors influencing adsorption were studied: first, the composition of the solubilizer, next, compounds used as additions to the solutions, representing types of possible metabolites or drugs present in the blood streams, and finally, the solid adsorbents corresponding to components of the intima.

Let us first consider the solubilizer. The bulk of the experiments was carried out with the cleared solutions described in the previous paper. Solubilization of cholesterol by soap type substances has been reported by others, using, for instance, polymerized nonionic soaps.[6] The solubilizers used by the present writer are salts of aliphatic acids with organic amines, hence, low molecular weight compounds. The characteristics of the micelles: aggregation number, fractional charge, and electrical mobility, among others, were previously determined and found comparable to data listed in the literature[6] for micelles built of aliphatic compounds of from 10 to 12 carbon atoms. The complexing ability of aliphatic acids for cholesterol, besides that of ester formation, may be seen also from clinical evidence: thus heparin that produces lipase in vivo and splits triglycerides enhances the excretion of cholesterol.[7] In our study nonanoic acid was used in the majority of tests, in a

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few experiments the unsaturated undecenoic acid was used for comparison. The two showed significant differences—see later. In a few additional experiments human beta globulin was used as solubilizer, forming chylomicrons, instead of cleared solutions.[8]

Several compounds were used as additions to the solutions which will be discussed later in connection with the results obtained. These compounds were, among others, methylene blue and congo red, two typical dyes, one cationic, the other anionic; further sodium dodecyl sulfate and sodium phosphates.

The various solids used as adsorbents will also be discussed later. These were: activated charcoal, blood fibrin and several calcium salts, besides a few others of lesser significance.

Experimental technique. A certain amount, mostly 20 mg, of the adsorbent was mixed with 1 cc of the solution prepared with C^{14} labeled cholesterol, and left for 30 minutes with intermittent shaking. After that time the mixture was filtered and the solid retained by the paper washed several times with water. The paper was then dried at about 90 C for 30 minutes, and the solid collected in a planchet. After further drying, the solid was spread as uniformly as feasible, and its radioactivity due to the labeled cholesterol measured.

Usually, adsorption from a solution is determined by measuring the concentration of the solute before and after adsorption. This method was not found practical in the present instance, especially for small amounts of adsorbate, since it involves the difference of two data of similar magnitude. It was therefore preferred to measure the activity of the adsorbate directly on the solid. Such a method also involves some uncertainty, namely that due to self-absorption of the soft beta radiation within the solid layer, yet this inaccuracy was deemed less than that of the difference method. In order to account for self-absorption, the measured activity was multiplied by a factor which is a function of the surface density of the layer: the mass of the solid adsorbent divided by the area of the layer. Fig. 1 gives these factors, calculated from theory.[9] In the majority of tests the factor was between 1.5 and 2.0. The mass of the solid in the planchet was accurately known, the area was estimated as closely as feasible. The applicability of Fig. 1 may be seen from the data shown in Table 1. A small amount, 0.04 cc of a cholesterol solution in ethanol, having, after evaporation of the solvent, an activity of 124 counts per minute, was mixed with known amounts of solids. The roughly circular

Table 1
Activity of adsorbent layers, corrected by means of factors of Fig. 1. Activity without solids: 124 counts per min.

Mass of solid, mg	Diameter of layer, mm	Surface density, mg/cm²	Factor from Fig. 1	Measured activity, c/m	Corrected activity, c/m
10	17	4.4	1.7	66	112
15	18	5.9	2.0	63	126
20	16	10	2.9	36	105

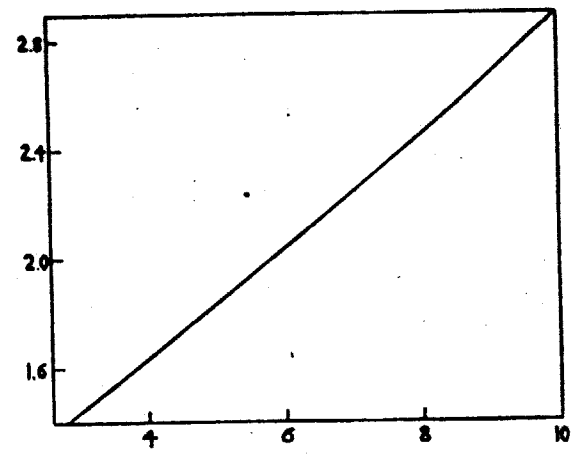

Fig. 1. Correction factors for self-absorption of C^{14} containing layers as function of surface density, mg/cm².

areas of the layers were estimated and the correction factors read from Fig. 1. The measured activities were multiplied with these factors; the corrected figures shown in the last column are in fair agreement with the activity of the solution used.

Washing of the residue of filtration was necessary in order to remove all activity from adhering solution. It was established that after washing a few times the activity measured did not diminish in spite of repeated rapid washings; we thus correctly measured the amount of adsorbed cholesterol.

The adsorption time of 30 minutes was chosen arbitrarily, for practical reasons. That time was not long enough for obtaining true equilibrium values of adsorption. In one case, for example, the following data were obtained: after 15 minutes of adsorption, 284 counts per min; after 30 minutes, 326 c/m; and after 60 minutes, 408 c/m. While the 30-minute data are not equilibrium values, they are satisfactory for the relative determinations intended; they may, in fact, reflect more closely conditions in vivo, where deposition of lipids is more a dynamic than a static process. We will come back to this matter presently.

Experimental results and discussion. The experimental results are now presented, taking first activated charcoal as adsorbent. This substance may be considered as representative of organic material generally. Since its adsorptive power is high, data obtained under different conditions vary within wide limits, and are therefore sufficiently informative. The first two columns of Table 2 give the solubilizers and their concentrations in m*M*. They yield cleared solutions, except the last group of three lines, referring to artificial, chylomicron type lipoproteins mentioned above.[8] The next column contains

the concentration of cholesterol in mg 100 cc. The following two columns give the names of added compounds with their concentrations in m*M*. The last column in Table 2 shows the percentage of cholesterol adsorbed by 20 mg of charcoal with reference to the total cholesterol present in 1 cc of solution. This figure was calculated by first multiplying the measured activity with the pertinent factors from Fig. 1, as illustrated in Table 1, and further multiplying the product by 20/m, where m is the mass in mg. of the dried charcoal present in the planchet. It is hereby assumed that the amount of adsorbate is small compared with that of the charcoal.

The usual manner of evaluating adsorption data differs from this one. When equilibrium has been reached, the amount of adsorbate is by theory a function of the final, equilibrium concentration. Recording our data in terms of these two significant quantities was not justified, since, as mentioned, equilibrium was not always reached. If wanted, however, our data can be converted to the two significant quantities mentioned. The amount of adsorbate in mg per g of adsorbent is given by 0.005 pc, where p is the percentage adsorption as listed and c the initial concentration of cholesterol as shown in Table 2; the final concentration of cholesterol is then given by (1−0.01 p)c. Thus, for line 1 of Table 2, the adsorbate is 11 mg per g of charcoal, and the final concentration of cholesterol is 18 mg per 100 cc.

The adsorption of cholesterol from its cleared solution takes place via its nonanoate vehicle. This acidic compound is primarily adsorbed and carries with it the solubilized cholesterol which also is deposited on the solid surface. The adsorption of the lipid is thus a secondary process. The adsorption of the aliphatic acid is governed, like that of any other compound, by two characteristic forces: an electric force due to the charge of the solid surface, and the van der Waals forces which find expression in the specific adsorptivity, depending on the chemical nature of both solute and adsorbent. It is not always possible to tell in each case which of the two forces is predominant.

Dyes in particular are known to impart their electric charge to the surface, thus they are likely to influence the adsorption of nonanoate-cleared cholesterol. A negatively charged surface, such as the majority of surfaces in contact with water, will acquire an increased negative charge by acidic dyes, and will reverse its charge, becoming positive, in the presence of basic dyes. The effect of such dyes is seen in the first six lines of Table 2. The first line gives the extent of adsorption without any addition, 54 per cent in 30 minutes. Experiments with added compounds were carried out in two different ways. In one, the adsorbent was in contact with the solution of the added compound for 30 minutes, after which time the solubilized cholesterol was added. In the other method, cholesterol and the added compound was mixed simultaneously with the adsorbent. The two methods yielded comparable results; the second seems preferable, as its conditions are closer to those prevailing in vivo when metabolites or drugs are present in the blood stream together with the lipoproteins.

Methylene blue, a basic dye, renders charcoal positive and increases the

Table 2
Adsorption by charcoal (20 mg) of cholesterol from 1 cc of solution in 30 min.

Solubilizer	Concentration of solubilizer, m*M*	Concentration of cholesterol, mg/100	Added compound	Concentration of added compound, m*M*	Percentage adsorption
TEA[a]-N[b]	50	40	–	–	54
TEA-N	50	40	Methylene blue	0.8	71
TEA-N	50	40	Congo red	0.2	54
TEA-N	50	40	Congo red	0.4	41
TEA-N	50	40	Congo red	0.8	19
TEA-N	50	40	Congo red	1.6	1.3
TEA-N	50	40	Na cholate	40	36
TEA-N	50	40	Na glycocholate	40	20
TEA-N	50	40	Na taurocholate	40	23
TEA-N	50	40	Na dodecyl sulfate	6	41
TEA-N	50	40	Na dodecyl sulfate	12	19
TEA-N	50	40	Na dodecyl sulfate	25	3.3
TEA-N	50	40	Na dodecyl sulfate	50	2.0
TEA-N	50	40	KH_2PO_4	80	57
TEA-N	50	40	Na_2HPO_4	80	66
TEA-N	50	40	$Na_4P_2O_7$	80	72
Ch[c]-N	50	40	–	–	9.0
Ch-N	50	40	Methylene blue	1.6	14
Ch-N	50	40	Congo red	1.6	2.0
Ch-N	50	40	Na dodecyl sulfate	50	2.4
TEA-U[d]	50	40	–	–	22
TEA-U	50	40	Na dodecyl sulfate	12	7.5
TEA-U	50	40	$Na_4P_2O_7$	80	54
Gl[e]	0.05	15	–	–	8.5
Gl	0.05	15	Na dodecyl sulfate	14	2.0
Gl	0.05	15	$Na_4P_2O_7$	50	9.0

a–Triethanolamine; b–Nonanoate; c–Choline; d–Undecenoate; e–beta globulin

adsorption of cholesterol; congo red, an anionic dye, makes charcoal more negative and reduces the adsorption of the nonanoate-solubilized lipid. The effect of congo red with increasing concentration is shown by the figures given in the table. At a concentration of 1.6 m*M* it practically suppresses the adsorption of the lipid. Since the pH of the blood is toward the alkaline side of the isoelectric point of most serum proteins, the latter are present in the form of their anions, and the effect of charge of the intima on the adsorption of lipoproteins is probably similar to that shown for nonanoate as stabilizer.

The effect of bile salts is in the same direction, although to a lesser extent than that of dyes, as may be seen from the three next figures. It is likely that in these instances the relative adsorptivities[10] of the nonanoate and cholate anions play a role. This is also the case with sodium dodecyl sulfate as addition. Its effect with increasing concentration in suppressing the adsorption of cholesterol is shown in the next four lines of the table: at 50 m*M*

suppression is virtually complete, the sulfated 12-carbon chain competing successfully with nonanoate.

An instance in which specific adsorptivities counteract the effect of electric charge is seen from the addition of phosphates in the following three lines of Table 2. Although the trivalent PO_4 and the quadrivalent P_2O_7 charge the charcoal particles negative, their specific attraction for nonanoate causes the adsorption to increase beyond the value found in the absence of added compounds.

The next four lines refer to choline nonanoate as stabilizer. Without addition, the percentage adsorption is only 9. The effect of dyes and dodecyl sulfate are in the same direction as with triethanolamine. Choline nonanoate is of interest in that both the base and fatty acids are normal body constituents. Choline appears to contribute noticeably to the binding of cholesterol by the organic salt, resulting in reduced adsorption of the lipid at solids in contact with the solution.

Of particular interest are the next three lines in which the stabilizer acid is the unsaturated undecenoic acid. Adsorption without added compound is here 22 percent, dodecyl sulfate reducing the amount, and sodium pryophosphate increasing it, as above. It appears that binding of cholesterol by an unsaturated acid with its pair of mobile pi electrons is stronger than by a saturated acid. As a result, the adsorption distribution will favor the aqueous phase with its excess of unsaturated acid, and adsorption will be reduced. It is not impossible that this effect is one of the factors in the beneficial role of unsaturated fatty acids in atherosclerosis.

The last three lines in Table 2 refer to chylomicron type lipoprotein solutions of cholesterol, prepared with human beta globulin. Here again the adsorption without added compound is low, only 8.5 percent. Pyrophosphate appears to have no effect, dodecyl sulfate again suppresses adsorption. It is to be expected that protein has a higher complexing power for cholesterol than the amine salts of aliphatic acids. Another factor in this connection is that, under comparable conditions, larger particles have lesser adsorptivity than finer dispersions approaching molecular dimensions. The observed difference in adsorption according to size of the micelles is in agreement with the views of Eyring on this matter.[11] It is also known that in the blood of atherosclerotic patients mostly the lipoprotein fraction of S_f 0 to 12 is elevated;[3] these are medium size particles of about 200 A diameter, appreciably smaller than chylomicrons. While the latter are not markedly involved in the formation of atheromata, they are mainly responsible in the clogging of arterioles of the myocardium, leading to angina pectoris. The action of heparin in relieving the symptoms within a few minutes[12] and reverting myocardial blood flow to normal levels[13] is attributable to the splitting up of chylomicrons clogging the vessels. In the organization of atheromas by adsorption they are however of lesser importance than the smaller, cleared lipid particles.

Let us next turn to blood fibrin as adsorbent, a compound that is actually present in atheromas, originating from thrombi. Fibrin deposition is then the

primary process; if fibrinolysis is inadequate, thrombi may organize.[3] The data shown in Table 3 refer to 25 mg of fibrin. The extent of adsorption is less than for activated charcoal, as is to be expected. Basic dyes increase the adsorption, acidic dyes reduce it, as does sodium dodecyl sulfate. Pyrophosphate has no influence on the adsorption. Choline nonanoate is adsorbed to a lesser degree than triethanolamine nonanoate, and the chylomicron type of lipoprotein shows no adsorption under the conditions of the experiment.

Table 3

Adsorption by 25 mg of blood fibrin of cholesterol from 1 cc of solution in 30 min.

Solubilizer	Concentration of solubilizer, mM	Concentration of cholesterol, mg/100	Added compound	Concentration of added compound, mM	Percentage adsorption
TEA-N	50	40	—	—	7.0
TEA-N	50	40	Methylene blue	1.6	20
TEA-N	50	40	Basic fuchsin	1.6	9.5
TEA-N	50	40	Congo red	1.6	3.7
TEA-N	50	40	Eosin	1.6	3.5
TEA-N	50	40	Na dodecyl sulfate	6	6.0
TEA-N	50	40	Na dodecyl sulfate	12	4.4
TEA-N	50	40	$Na_4P_2O_7$	80	6.5
Ch-N	62	50	—	—	2.1
Ch-N	62	50	Basic fuchsin	1.6	5.2
Ch-N	62	50	Eosin	1.6	2.0
Gl	0.05	15	—	—	0.0

Besides fibrin, an adsorbent of the protein group, it seemed of interest to investigate a carbohydrate and a triglyceride type of adsorbent. The former, a cellulose, gave no measurable adsorption, while the latter, trilaurin, gave adsorption data of the order of magnitude of only 2 percent.

Of greater importance than these are calcium salts, to be discussed next. Atheromas are known to calcify and can contain varying amounts of calcium salts. The adsorptive power of these latter is high; also, it is known that the adsorption of sodium dodecyl sulfate on charcoal increases with increasing ash content.[14] As the data of Table 4 show, the percentage adsorption of cholesterol on calcium phosphate is appreciable. The first three lines refer to triethanolamine nonanoate in increasing concentrations, that of the lipid increasing proportionately. From the general form of adsorption isotherms (adsorbate *vs* concentration) decreasing percentage of adsorption with increasing concentration follows; as the figures show, that relation holds here too. This is significant with regard to our finding that from chylomicron type solutions adsorption of cholesterol is less than from cleared solutions. The concentration of cholesterol in the former was 15 mg/100, in the latter 40 mg/100, hence the percentage adsorption from the former is expected to be higher than in the latter; yet, for reasons given above, the opposite is found.

Table 4

Adsorption by 20 mg of $Ca_3(PO_4)_2$ of cholesterol in 30 minutes

Solubilizer	Concentration of solubilizer, mM	Concentration of cholesterol, mg/100	Added compound	Concentration of added compound, mM	Percentage adsorption
TEA-N	20	16	–	–	85
TEA-N	50	40	–	–	51
TEA-N	125	100	–	–	32
TEA-N	50	40	Methylene blue	1.6	82
TEA-N	50	40	Na dodecyl sulfate	12	41
TEA-U	50	40	–	–	22
TEA-U	50	40	Methylene blue	1.6	39
TEA-U	50	40	Congo red	1.6	11
GI	0.05	15	–	–	38
GI	0.05	15	Methylene blue	1.0	52
GI	0.05	15	Congo red	1.0	25
GI	0.05	15	Na taurocholate	25	31
GI	0.05	15	Na dodecyl sulfate	12	5.5

The unsaturated undecenoate shows smaller figures than nonanoate, as with charcoal, in possible correlation with the beneficial role of unsaturated fatty acids in the treatment of atherosclerosis. In combination with a compound, like congo red, that renders the adsorbent strongly negative adsorption is reduced to a low figure, 11 percent. As just mentioned, adsorption from chylomicron type lipoproteins is less than from cleared ones; in combination with dodecyl sulfate adsorption is only 5.5 per cent.

Table 5 gives data for calcium stearate, a salt formed from calcium and a fatty acid, and Table 6 presents data for calcium oxalate. Essentially, the results correspond to those obtained with the other adsorbents. Of special interest are the low figures, 1.1 per cent, for calcium stearate (Table 5) when

Table 5

Adsorption of cholesterol by 20 mg of calcium stearate in 30 minutes.

Solubilizer	Concentration of solubilizer, mM	Concentration of cholesterol, mg/100	Added Compound	Concentration of added compound, mM	Percentage adsorption
TEA-N	50	40	–	–	14
TEA-N	50	40	Methylene blue	1.6	70
TEA-N	50	40	Na dodecyl sulfate	12	1.1
TEA-U	50	40	–	–	1.4
TEA-U	50	40	Methylene blue	1.6	12
TEA-U	50	40	Na dodecyl sulfate	12	1.2
GI	0.05	15	–	–	5.3
GI	0.05	15	Methylene blue	1.0	8.2

sodium dodecyl sulfate is added to 50mM triethanolamine nonanoate, and the equally low adsorption, 1.4 per cent, for triethanolamine undecenoate without any further addition. These results are noteworthy in that adsorbents such as calcium stearate, formed from fatty deposits by subsequent calcification, are probably present in atheromas.

Table 6

Adsorption of cholesterol by 20 mg of calcium oxalate in 30 minutes. Concentration of solubilizer: 50 mM, that of cholesterol: 40 mg/100.

Solubilizer	Added compound	Concentration of added compound, mM	Percentage adsorption
TEA-N	–	–	25
TEA-N	Methylene blue	1.6	52
TEA-N	Congo red	1.6	13
TEA-U	–	–	7.3

SUMMARY

The adsorption of cholesterol by solids from aqueous solutions was studied, it being assumed that adsorption plays a certain role in the formation of atheromata. The following results were obtained:

1. Adsorption is appreciable in the case of activated charcoal and water-insoluble calcium salts, phosphate and stearate, and somewhat less for blood fibrin as adsorbents. Calcium salts as well as fibrin are present in atheromas.
2. Adsorption is high from cleared cholesterol solutions, and less from chylomicron type lipoprotein solutions. This finding is in agreement with the fact that lipoprotein particles of S_f 0 to 12 are mainly elevated in the sera of atherosclerotic patients.
3. Basic dyes, such as methylene blue, strongly increase adsorption, while acidic dyes, such as congo red, reduce it. This is explainable on the basis that the cholesterol particles are negatively charged.
4. Certain compounds, such as sodium dodecyl sulfate, strongly reduce and even completely suppress the adsorption of cholesterol. This is due to the competing effect of the alkylated sulfate anion with the cholesterol-aliphatic acid complex.
5. Cleared solutions prepared with the unsaturated undecenoate show a noticeably lesser degree of adsorption than those prepared with the saturated nonanoate. This finding might partly explain the beneficial role of unsaturated fatty acids in atherosclerosis.

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THE EFFECTS OF QUIET STANDING ON SOLUTE DIURESIS [1]

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INTRODUCTION

In normal supine human subjects (1, 2), the rapid intravenous injection of glucose or mannitol sharply augments the excretion of sodium. Animal experiments indicate that the natriuresis may be mediated at least in large part by a direct renal response to a change in the concentration of the solute in the blood perfusing the kidney (3). It has been suggested that the injected solute restrains the reabsorption of water in the proximal tubules and interferes with the reabsorption of sodium by accelerating the flow of the tubular fluid (1, 3), or by diluting the concentration of tubular sodium below that in extracellular water (4). It is implied in these theories that the capacity of the tubular system for sodium transport is either fixed, or only slowly adaptive to changing loads of sodium. It has therefore been of considerable interest to determine the extent to which, and the rapidity with which, internal regulatory factors may counterbalance the intratubular effects of administered solutes. Certain measures which promote the retention of sodium in normal subjects (antecedent salt restriction, or the administration of DOCA) are comparatively ineffective in preventing the sodium diuresis induced by solute loads (5). However, internal regulatory mechanisms in patients with edema severely limit the excretion of sodium despite massive glycosuria (6) even when the renal hemodynamic pattern is essentially normal. It is not clear, therefore, whether the retention of sodium during osmotic diuresis is a unique feature of the edematous state, or whether the stimuli which have been used to promote salt retention in normal subjects are of insufficient intensity.

Since *quiet standing* constitutes an extremely potent stimulus for sodium and water retention (7–9), the present experiments were undertaken with the following objectives in mind: (*1*) to determine whether normal subjects, under the intense stimulus to retain sodium imposed by quiet standing, nevertheless develop the usual diuresis of sodium with solute loads; (*2*) to decide, by means of clearance techniques, whether the effects observed are mediated by changes in glomerular filtration or tubular transport; and (*3*) to identify, where possible, the changes in the internal environment which may have been conditioning the renal response.

EXPERIMENTAL PROCEDURE

Control and experimental studies were carried out in nine young healthy male subjects. Urine and blood were first obtained while the subject was engaged in normal activity (expts. 1 and 2—Table I), or lying supine on an examining table (expts. 2 to 14—Tables I and II). Next, several samples of blood and urine were collected during *quiet standing* (the subject stood at the side of a shelf on which his arms rested, with only such minimal movement of the trunk and extremities as was found necessary to prevent collapse). Finally, several recovery periods were obtained during which the subject again assumed a supine position. In one experiment (Table II, expt. 9) additional periods during quiet standing and recovery in the supine position followed. Urine was collected in all cases by voluntary voiding. In our experience, the error involved in this method, in selected normal young subjects, is small. The possible effect of such an error on the urinary changes observed between periods of low and those of high flow, has been minimized in most cases in these experiments by averaging the data of more than one period of actual urine collection in each of the "periods" designated *A, B, C, etc.*, in Tables I and II.

Two types of control studies are summarized in Table I. The effects of quiet standing alone, without solute loading, were studied in three experiments during dehydration and hydration. Table I summarizes the effects of injections of hypertonic solutions of sodium bicarbonate and sodium phosphate (pH 7.4) while the subject was supine. Table II summarizes the results of experimental studies of the effects of injections of mannitol and sodium salts during the period of quiet standing.

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Data, in part previously reported by this laboratory (2, 9), on the effects of mannitol loads in supine subjects, and of saline infusions in both supine and standing subjects, have also been used in the construction of Table III and Figures 1 and 2.

In six experiments (expts. 3, 8, 10, 11, 12, 13), glomerular filtration rates were estimated from the clearance of inulin administered as a constant infusion. The isotonic sodium phosphate used in experiments 4 and 14 was prepared as a mixture of NaH_2PO_4 and Na_2HPO_4 of pH 7.4 (10).

Mannitol in serum and urine was analyzed by the method of Smith (11), modified according to Elkinton (12), inulin by the method of Roe (13), potassium and sodium on a flame photometer, and inorganic phosphate by the method of Fiske and Subbarow (14) as modified for the photoelectric colorimeter. Chloride in the serum was determined by the method of Hald (15), protein by the biuret reaction (16), and total CO_2 content by the method of Van Slyke and Neill (17). Urea and ammonia in the urine were determined by the methods of Conway (18), and chloride by a modification of the method of Volhard and Harvey (19). Acute changes in plasma volume were calculated from changes in the hemoglobin and hematocrit as described previously (9). The pH of serum was determined, with anaerobic precautions, with a glass electrode (Beckman). Because of the difficulties of collecting urine anaerobically under the conditions of these experiments, bicarbonate concentrations in the urine were not directly measured. However, since bicarbonate constitutes the major urinary anion in those experiments in which sodium bicarbonate was infused, changes in the excretion of bicarbonate were estimated from changes in the excretion of "undetermined anions" (calculated according to the formula: $A = [Na + K + NH_4] - Cl$, where A is the rate of excretion of "undetermined anions"). In 27 urines collected anaerobically in normal individuals during the infusion of sodium bi-

TABLE I

Control studies. The effects of quiet standing, and of infusions of $NaHCO_3$ and Na_2HPO_4, on the serum and urine of normal subjects

Subject*	Procedure	Period†	Time	Urine: Flow	Urine: Na	Urine: Cl	Urine: K	Urine: NH_3	Urine: P‖	Urine: Anions‖	GFR	Serum: Na	Serum: Cl	Serum: K	Serum: CO_2	Serum: Prot.	Serum: pH	Serum: P‖	Change in plasma vol.§
				cc./min.	micro. Eq./min.						cc./min.	mEq./liter				gms. %		mg. %	per cent
1) AVG (a)	Ambulant	w.	mins.	0.6	44.4	68.0	39.6	26.0				142	101	3.6		7.75			0
	Standing	A	140	0.2	4.7	15.6	27.2	19.5				138	104	3.6		7.95			−12
	Supine	B	63	0.3	6.8	19.1	38.3	21.3				*138*	*106*	*3.7*		*7.05*			+ 5
		C	131																
2) DWS (a)	Ambulant	A	74	0.6	108	125	37.4	16.7	13.3			138	102	3.4	27.3	7.92	7.43	4.8	0
	Supine	B	64	0.6	140	100	42.5	13.3	23.5			140	102	3.5	27.0	7.25	7.46	4.6	+14
	Standing	C	38	0.4	81.2	92.0	38.6	8.0	16.6			137	102	3.8	26.0	8.18	7.49	3.8	− 6
	Supine	D	50	0.2	9.1	12.2	19.0	6.6	11.6			*142*	*102*	*3.5*	*27.1*	*7.54*	*7.47*	*4.2*	+ 2
	Supine	E	69	0.3	18.8	27.0	23.1	8.1	25.8										
3) DWS (b)	Supine	A	66	6.8	294	403	102	41.7	13.5		161	140	105	5.0		6.54		3.5	
	Standing	B	69	1.4	210	270	70.0	37.5	12.7		126	141	105	4.9	25.0	6.78	7.42	3.6	0
	Supine	C	32	1.1	160	230	57.0	38.3	18.7		103	141	105	5.0	22.4	7.22	7.38	3.6	−24
	Supine	D	86	4.7	247	318	78.5	39.9	31.7		*140*	*140*	*105*	*5.0*	*24.2*	*6.20*	*7.48*	*3.8*	+ 4
4) H., 1 liter, 1% NaH_2PO_4	Supine	A	114	3.1	91.5	83.2	120	16.4	53.4			139	96.0	4.3	26.7	7.12	7.46	4.5	
	Supine	B**	116	3.8	161	24.2	210	27.3	472			131	93.0	3.8	25.9	6.71	7.32	10.1	
	Supine	C	91	4.0	248	25.3	203	21.6	384			138	93.3	3.6	26.3	7.00	7.49	10.2	
5) Mac, 400 cc., 3.1% $NaHCO_3$	Supine	A	108	0.6	79.6	122	34.4	32.8		25		132	103	4.7	24.4	7.20	7.44		
	Supine	B**	34	2.6	527	179	191	17.0		556		145	98.4	4.3	31.8	6.28	7.52		
	Supine	C	146	1.6	371	51.8	151	1.6		471		142	101	4.8	29.1	7.16	7.46		
	Supine	D	225	0.8	88.7	39.5	8.4	7.1		74									
6) Kat, 350 cc., 2.7% $NaHCO_3$	Supine	A	66	1.1	172	199	91.5	38.6		104		140	103	4.3	24.1	7.38	7.43		
	Supine	B**	104	2.0	377	206	200	11.5		382		144	99.7	3.9	28.0	7.50	7.46		
	Supine	CW	210	1.2	253	88.5	134	<1		298									
7) Hal, 400 cc., 3.1% $NaHCO_3$	Supine	A	98	2.7	200	259	113	26.6		120		132	95.0	5.7	27.6	7.82	7.30		
	Supine	B**	63	6.2	835	330	249	5.5		759		139	94.0	5.4	32.3	6.96	7.32		
	Supine	C	89	2.8	373	127	193	2.1		441		136	93.6	5.1	32.0	7.05	7.40		
	Supine	DW	140	2.2	162	35.8	103	3.4		232									

* Subjects were normal young adults. AVG, DWS (a), Mac, and Kat were thirsting for 12 to 14 hours before the experiments, and were given no water during the experiments except in period marked w. DWS (b), H, and Hal were allowed water *ad lib.* before the experiments, and drank 100 cc. per hour during the experiments. DWS (b) ingested 15 gms. of NH_4Cl in 36 hours before experiment. This subject also was infused with normal saline containing requisite inulin and sodium para-aminohippurate, at about 2.3 cc. per minute throughout the experiment.

† Infusions of test solutes were given during periods marked**. Water p. o. allowed *ad lib.* in periods marked w. Each period composed of 1 to 3 sub-periods corresponding to individual collections of urine.

‡ Serum values at the beginning of each corresponding period, except for values *in italics* which are at the end of the corresponding period.

§ Changes in plasma volume at the beginning of each corresponding period, calculated with respect to the control period, which is indicated by 0.

‖ P calculated as B_2HPO_4.

‖ See Experimental Procedure for calculation of *anions*.

TABLE II

The infusion of mannitol, $NaHCO_3$, and Na_2HPO_4 into normal subjects during quiet standing

Subject*	Procedure	Period‡	Time	Urine								GFR	Serum‡								Change in plasma vol.§
				Flow	Na	Cl	K	NH_4	Pi‖	Animal‖	Mann.		Na	Cl	K	CO_2	Prot.	pH	P	Mann.	
		no.	*min.*	*cc./min.*	*micro. Eq./min.*						*micro. mol/min.*	*cc./min.*	*mEq./liter*				*gm. %*		*mg. %*	*mM/liter*	*per cent*
8) DWS (c), 350 cc., 25% mannitol	Supine	A	138	0.9	207	171	73.4	6.15				112	139	105	4.0		6.68				0
	Standing	B**	51	4.9	384	347	77.0	9.43			1,930	110									
	Standing	C	24	6.8	338	344	65.0	11.6			3,560	123	123	97.1	4.4		6.58			656	+ 5
	Supine	D	87	4.1	215	219	64.6	12.0			1,925	118	*128*	*102*	*4.0*		*6.15*			*92*	*+ 8*
9) AVG (b), 350 cc., 25% mannitol	Supine	A	53	0.9	133	186	87.0	23.0					145	105	3.9		6.90				0
	Standing	B**	29	6.9	320	399	106	31.6			2,255		*137*	*100*	*4.2*		*6.46*			*372*	*+10*
	Supine	C	35	5.2	293	368	115	18.6			1,700		136	99.2	4.7		7.10			279	− 3
	Standing	D	43	3.7	250	352	120	20.0			1,138		138	104	4.2		6.53			221	+ 7
	Standing	E	60	1.9	52.5	128	57.3	37.2			636										
	Supine	F	51	1.7	90.6	149	67.0	22.2			513		140	105	4.0		7.65			130	−18
10) AVG (c), 1 liter, 4.25% mannitol	Supine	A	123	10.9	172	117	93.7	1.54	49.9			108	140	101	3.9		7.45		3.8		0
	Standing	B**	80	4.1	105	83.5	29.4	37.4	5.2		787	101	141	100	4.2		7.01		3.2	229	+ 7
	Supine	C	62	12.7	93.1	56.9	28.3	47.1	18.6		419	102	131	93.3	4.6		7.15		3.6	146	+12
	Supine	D	107	9.6	148	80.0	22.6	30.8	31.7		150	106	*140*	*101*	*4.6*		*6.68*		*4.3*	*16*	+18
11) JCR, 525 cc., 3.5% $NaHCO_3$	Supine	A	163	1.3	127	160	102	24.7		48		167	*141*	*104*	*4.0*	*25.2*	*6.50*	*7.37*			*0*
	Standing	B**	47	1.7	329	140	240	9.2		423		195	*142*	*101*	*3.3*	*32.2*	*6.08*	*7.39*			*+11*
	Supine	C	63	1.6	328	67.5	257	1.3		517		141	*140*	*101*	*3.6*	*29.4*	*6.20*	*7.38*			*+ 9*
12) AVG (d), 525 cc., 3.5% $NaHCO_3$	Supine	A	188	5.3	141	179	179	30.5	26.6	171		155									
	Standing	B	27	7.7	54.6	77.0	85.5	47.5	17.5	112		131	139	103	4.4	27.3	6.82	7.40	2.9		0
	Standing	C**	85	1.3	242	41.5	150	7.2	23.7	358		165	145	102	4.2	31.6	7.16	7.43	3.0		− 6
	Supine	D	113	3.0	395	28.9	159	5.5	59.6	526		170	*142*	*99.4*	*3.9*	*32.0*	*6.07*	*7.43*	*3.2*		*+22*
13) AB, 525 cc., 3.5% $NaHCO_3$	Supine	A	70	8.6	199	185	73.1	20.8		112		223(?)									
	Standing	B**	65	3.5	374	204	110	13.0		293		160	140	101	3.9	25.8	6.58	7.35			0
	Supine	C	35	2.9	661	145	193	1.2		710		145	144	96.0	4.3	33.9	6.15	7.30			± 0
14) AVG (e), 1 liter, 1% Na_2HPO_4	Supine	A	147	5.8	67.8	119	146	20.8	53.6				145	102	4.4	28.7	6.77	7.42	3.6		0
	Standing	B**	94	3.6	273	22	151	19.0	568				*149*	*96.6*	*3.7*	*28.4*	*6.87*	*7.40*	*17.1*		*−10*
	Supine	C	137	5.7	316	<10	133	15.1	487				*146*	*98.3*	*3.6*	*29.6*	*6.82*	*7.42*	*6.9*		*+ 6*

* DWS (c), AVG (b), and JCR were thirsting for 12 to 14 hours before the experiments, and during the experiments, except that JCR drank 250 cc. water 2 hours before the experiment. Other subjects were allowed water *ad lib.* before the experiments, and ingested 100 cc. per hour during the experiments.
See Table I for other footnotes.

carbonate, this estimate has been found to agree with the measured excretion of bicarbonate with an error of 18 ± 9 per cent.

RESULTS

Quiet Standing Alone (Table I):

The effects of quiet standing during hydration and water deprivation were studied in three experiments on two subjects. The passive erect position caused the plasma volume to contract. Urine flow, sodium, potassium and chloride excretion decreased in all experiments, and remained below control values during one or more periods in the supine position. Glomerular filtration decreased in the one experiment in which it was measured. In addition, the excretion of ammonia decreased slightly in the two hydropenic subjects, phosphate excretion was unchanged or declined in two experiments, and the serum bicarbonate decreased slightly, with no significant change in the serum pH in the two experiments in which it was measured.

Quiet Standing with Mannitol Infusion (Table II):

The administration of 350 cc. of 25 per cent mannitol to two thirsting subjects (expts. 8 and 9) prevented any significant contraction of the plasma volume. Presumably the transudation of fluid in the lower extremities caused by quiet standing was balanced by the movement of fluid from the tissues into the blood stream, possibly as a result of two mechanisms: (*a*) the extraction of water from cells (indicated by the decrease in the serum sodium), and its distribution to the plasma volume as a part of the extracellular space; and (*b*) the withdrawal of fluid from the extracellular fluid into the blood, as a result of the transient osmotic gradient set up during diffusion of mannitol from the vascular compartment.

Glomerular filtration was also maintained at control values during the period of quiet standing in experiment 8.

The excretion of water, sodium, and chloride

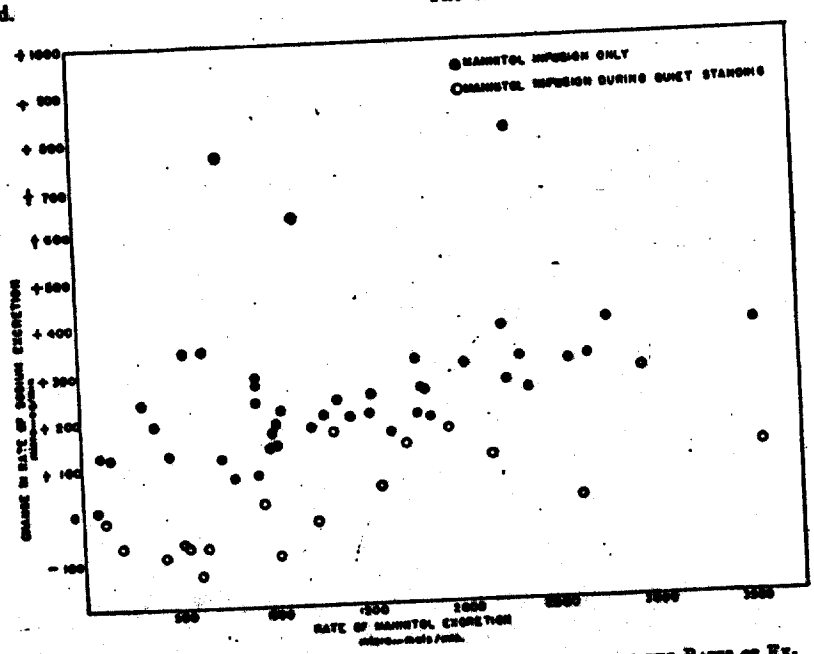

FIG. 1. CHANGES IN THE RATE OF EXCRETION OF SODIUM COMPARED WITH THE RATES OF EXCRETION OF MANNITOL CAUSED BY INFUSIONS OF MANNITOL

Individual periods of urine collection. Data concerning the infusion of *mannitol only*, taken from seven experiments in part previously reported (2, 20).

TABLE III

Modification of the decrease in sodium excretion during quiet standing by infusions of sodium salts

Infusion given during quiet standing	No. of observations	Mean change* in sodium excretion *micro. Eq./min.*	Range* *micro. Eq./min.*
None**	6	− 91	− 39 to −243
400 cc., 3-4% NaCl**	2	−101	− 30 to −172
400 cc., 6% NaCl**	4	− 41	+273 to −149
500 cc., 3.6% $NaHCO_3$	3	+140	+228 to +117
1,000 cc., isotonic, neutral sodium phosphate	1	+209	—

* Values calculated as the differences between the average rates of 1–3 control periods, and the rates of 1–2 experimental periods.

** Data of 3 of the experiments without infusions, and of all the experiments with NaCl infusions, were taken from another study (4).

increased during the infusion of mannitol and for several periods thereafter (expts. 8 and 9). However, it is evident from Figure 1 that quiet standing in these experiments limited the usual increase in excretion of sodium which is caused in the normal subject by the infusion of mannitol. Indeed, in one of the experiments (expt. 9, Table II), sodium excretion decreased below control values during a second period of quiet standing and then increased again when the subject was supine, although the excretion of mannitol continued slowly to decline.

In a third experiment, on a hydrated subject (expt. 10, Table II), the injection of a smaller quantity of mannitol maintained glomerular filtration at control values, and the plasma volume at or above control values, but sodium excretion declined below control values despite a mannitol diuresis which would have induced marked losses of sodium, if the subject had been supine.

Quiet Standing with the Infusion of $NaHCO_3$ or Na_2HPO_4:

Control Studies: The infusion of 18.75 gms. of $NaHCO_3$ as a 3 per cent solution into normal supine subjects augmented greatly the excretion of water, sodium, and potassium. Chloride ex-

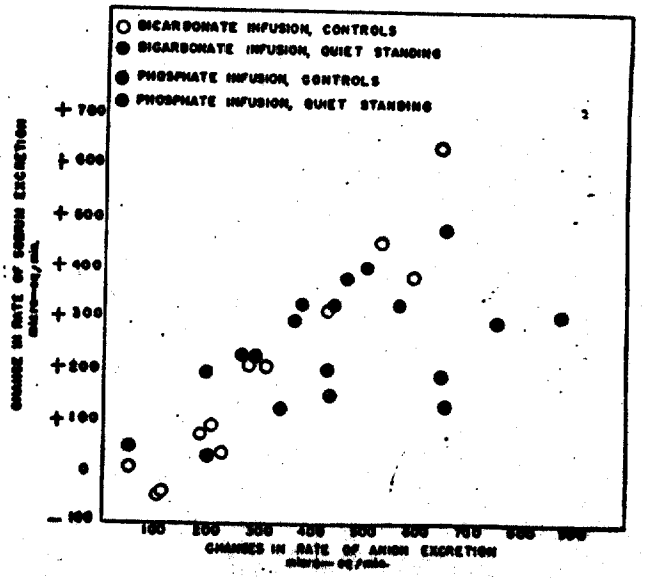

FIG. 2. CHANGES IN THE RATE OF EXCRETION OF SODIUM COMPARED WITH CHANGES IN THE RATE OF EXCRETION OF ANIONS CAUSED BY THE INFUSION OF $NaHCO_3$ AND Na_2HPO_4

Individual periods of urine collection.

cretion initially increased, but then decreased. Ammonia excretion uniformly declined. The concentrations of bicarbonate and sodium in the serum increased; those of chloride and protein decreased.

The infusion of one liter of isotonic Na_2HPO_4 into one normal subject increased greatly the excretion of sodium and potassium, increased slightly the excretion of ammonia, and decreased the excretion of chloride. The concentrations of chloride and protein in the serum decreased slightly, that of phosphate increased greatly.

Studies with Quiet Standing: Injections of sodium bicarbonate and buffered sodium phosphate prevented any significant fall of plasma volume, in three of four experiments (expts. 11, 12, 13), or of glomerular filtration rate in two experiments (expts. 11 and 12). However, sodium excretion was augmented in the same manner during quiet standing as in the supine position in each experiment (Figure 2). This was in sharp contrast to the inhibition of sodium excretion caused by the passive erect posture during mannitol loading (described above) and during salt loading (Table III). In the latter Table, various sodium salts are compared for their effects on the usual retention of sodium induced by quiet standing. Although the excretion of sodium decreased during quiet standing in spite of infusions of sodium chloride (except in one experiment with 6 per cent saline), it increased in all experiments in which sodium bicarbonate or phosphate was infused. Potassium excretion was augmented by the administration of sodium bicarbonate during quiet standing, as in control studies, but was not appreciably changed by infusions of sodium phosphate.

DISCUSSION

A. *Infusions of Mannitol*

In the present experiments on normal subjects during quiet standing, as in previous experiments on patients with edema (6, 20), the sodium diuresis induced by mannitol loads was partially inhibited (Figure 1). Since glomerular filtration rate remained at or above control values (expts. 8 and 10) the inhibition of sodium diuresis appeared to be the result of an increased capacity of the renal tubules to reabsorb sodium. This conclusion is in doubt only to the extent that the estimation of glomerular filtration by present clearance methods is not sufficiently accurate to define the exact relationship between a small (unmeasurable) fall in the filtered sodium and a change in the tubular reabsorption of this ion.

The results do not imply that the renal responses to infused solutes are not mediated at least in part by the direct effect of a change in the solute content and flow of the tubular fluid (3). They do emphasize, however, that internal regulatory mechanisms in normal subjects, as in patients with edema, may be of sufficient intensity to limit the intratubular effects of an administered solute on the reabsorption of sodium. What specific regulatory mechanisms are involved, and whether they may apply to the state of edema, are questions concerning which only inferential data are now available.

In the present experiments, several factors of the internal environment were considered possible stimuli for the retention of sodium:

1. Changes in the serum sodium (Table II), and calculated internal shifts of water and electrolytes were not significantly different during quiet standing from those in previously reported experiments in which mannitol was administered in the supine position (2). In experiments 8 and 9, when hypertonic mannitol was infused, intracellular balances of water and potassium were negative. Positive values for sodium were too small to be considered significant. In experiment 10, when 4.25 per cent mannitol was infused, intracellular balances of water, sodium, and potassium were not significantly altered.

2. Support of the total plasma volume in these experiments by the infusion of mannitol, as in other similar studies by the infusion of albumin (9), failed to eliminate the stimulus for salt retention (Figure 1) elicited by standing. These infusions, however, undoubtedly did not prevent local pooling of blood (in the lower extremities), transudation in the area of increased venous pressure, and consequent redistribution of the flow and volume of the circulating blood. The importance of these factors in the control of salt excretion may be inferred from studies of other abnormal circulatory states in which they may also be operative: Sodium retention occurs with the venous congestion produced by tourniquets on the limbs (21)

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or by obstruction of the inferior vena cava (22, 23) and with the portal congestion of decompensated hepatic cirrhosis (24) just as it does when a large volume of blood is actually removed from the circulation by hemorrhage (25). With venous obstruction, cardiac output and arterial blood pressure tend to fall (26). The renal response occurs in spite of lumbodorsal sympathectomy (21) and is independent of the pituitary (27), the adrenals (28) or of measurable circulatory changes in the kidney, but it may be eliminated by very large blood transfusions (26) (which probably overcompensate for blood lost by pooling). The upright position causes vasoconstriction in the cerebral (29) and splanchnic (30) areas; the renal response may occur in the absence of the adrenals (31).

It is possible, therefore, that in the present experiments, although there was no hemoconcentration when mannitol was infused during quiet standing, the kidneys may have responded not to the circulating blood volume as a whole, but to a change in the flow and/or volume of blood in some "central" area of the circulation (32, 33).

B. *Infusions of Sodium Bicarbonate and Phosphate*

In order to determine more precisely the intensity and character of the increased tubular capacity for sodium transport which is induced by quiet standing, sodium bicarbonate and buffered sodium phosphate were injected in experiments 11 to 14. These salts impose unique limitations on the tubular reabsorption of sodium. When the anion of an administered electrolyte is reabsorbed only to a slight extent, the total excretion of cations into the urine is correspondingly increased because of the limited quantity of free acid which may be secreted by the tubules.[4] The factors which determine the nature of the "covering" cation are unclear, but under ordinary conditions, particularly when the administered electrolyte is a sodium salt, this cation is mostly sodium. In the presence of a strong stimulus for the retention of sodium, it might be expected that the increased cation would be largely potassium (or conceivably partly ammonium, in the case of phosphate or sulfate). Indeed, when sodium bicarbonate is administered to patients with congestive heart failure or with "decompensated" hepatic cirrhosis, the bicarbonate ion is excreted to a great extent with potassium (20). However, in the present experiments, injected sodium bicarbonate and sodium phosphate were excreted much more easily during quiet standing than were similar quantities of sodium chloride which were of about the same tonicity and which increased the serum sodium to approximately the same extent (Table III). Furthermore, standing did not alter the role of sodium as the chief "covering" cation in the urine (Figure 2). The increased excretion of potassium was similar to that which was observed in the absence of a stimulus for the retention of sodium.

Two explanations for these results suggest themselves: (*1*) the effects of administered phosphate or bicarbonate on the excretion of sodium involve renal tubular mechanisms which may be entirely distinct in location (proximal *vs.* distal) or character (ion exchange *vs.* trace resorption) from those involved in the sodium retention of quiet standing; or (*2*) the stimulus for sodium retention imposed by quiet standing is not sufficiently powerful to counteract the unique limitation imposed by the excretion of bicarbonate and phosphate on the reabsorption of sodium. It may be that the excretion of sodium bicarbonate would be curtailed in the normal subject, as it is in the patient with edema (20, 34–36), if the stimulus for the retention of sodium were intense enough and sufficiently prolonged.

[4] This limitation is imposed not only because of the extreme acidity of acids such as H_3PO_4 and H_2SO_4, but also, in the case of bicarbonate, because of the probability that CO_2 has a great capacity to diffuse back rapidly across the tubular membrane.

SUMMARY

Infusions of mannitol, sodium bicarbonate and sodium phosphate were administered to normal subjects in the supine position and during quiet standing.

Observations were made of changes in the plasma volume, the serum electrolytes, and the urinary excretion of the loading solute, and of sodium and other electrolytes.

It was found that quiet standing partially inhibited the sodium diuresis induced by the administration of mannitol, but did not interfere with the increased excretion of sodium caused by infusions of $NaHCO_3$ or Na_2HPO_4.

The results with mannitol indicate the operation of internal regulatory mechanisms on the intrinsic renal responses which characterize solute diuresis.

The results with sodium bicarbonate and phosphate are discussed in relation to the unique limitations imposed by these salts on the tubular reabsorption of sodium.

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THE EFFECTS OF SODIUM HEXAMETAPHOSPHATE (CALGON) ON THE COMPLEMENT ACTION OF SERUM.

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THERE is now a considerable volume of evidence to show that complement action is associated with the protein constituents of serum. That non-dialysable calcium compounds linked to the protein might have a bearing on this problem was suggested by Gordon, Whitehead and Wormall (1926b). Citrated plasma shown by Welch, Brewer and Hunter (1940) to possess complement activity, a fact also confirmed by ourselves, would, however, rather militate against the view that calcium is involved in this reaction.

Within the last few years sodium hexametaphosphate has become of increasing technical importance, as this material has the property of removing calcium and magnesium ions from solution and is therefore capable of removing the last traces of hardness in water. This chemical is marketed under the trade name of "Calgon," and a large quantity is now used for scouring and cleaning various textile fabrics. The view is generally held that the calcium, magnesium or indeed other metal ions are converted into complex anions of the type $[M_2(PO_3)_6]^{--}$, where M is a divalent metal. Another reaction given by hexametaphosphates is the formation of addition compounds with proteins, a well known example being the turbidity produced in acidified albumin solutions after addition of sodium hexametaphosphate solution. In fact it is possible to convert animal skin into a form of white leather by using Calgon in the presence of hydrochloric acid and common salt, the latter being used to check undue swelling of the skin by the acid.

As a consequence of these properties of sodium hexametaphosphate it was determined to investigate the effects of this chemical on the complement action of serum. The material employed was "Calgon T" in the form of glassy plates of about 1/16 inch in thickness, which dissolve easily in water to give a solution of about pH 6·2. Such solutions were invariably adjusted to pH 7·4.

EFFECTS OF SODIUM HEXAMETAPHOSPHATE AND CALCIUM IONS.

The first series of experiments were as follows: Quantities of 0·3, 0·4, 0·5 and 0·6 c.c. of 10 per cent. Calgon in distilled water and adjusted to pH 7·4 were added to 3·0 c.c. quantities of normal guinea-pig serum. The mixtures were then allowed to stand on the bench for 2 hours, after which quantities of 0·1 c.c. of the mixtures were added to 0·5 c.c. of a 4 per cent. suspension of

sensitized ox-cells in 0·85 per cent. sodium chloride. After incubation at 37° C. for two hours the degree of haemolysis was read. In this series of experiments it was noted that only partial haemolysis was obtained when 0·3 c.c. of Calgon solution was employed. With quantities higher than 0·3 c.c. no haemolysis was observed. These experiments show clearly that complement activity is removed by the addition of certain concentrations of Calgon to the serum. This may be due to either:

(*a*) The removal of calcium ions from the serum; or—

(*b*) The effect of the hexametaphosphate on the serum proteins.

In the second series of experiments, quantities of 0·2, 0·3, 0·4 c.c. of 2·5 per cent. calcium chloride solution adjusted to pH 7·4 were added to 1 c.c. quantities of those mixtures in which the removal of complement activity had been effected by the Calgon treatment after the period of two hours' standing on the bench. A heavy precipitate was noted with the tubes containing 0·3 and 0·4 c.c. of calcium chloride solution and only a slight one with the 0·2 c.c. tube. The tubes were centrifuged to remove the precipitate, and 0·1 c.c. of the clear solution added to 0·5 c.c. of the 4 per cent. suspension of sensitized ox cells. Haemolysis was complete in the cases where heavy precipitates had been noted. Where precipitation had not been complete, as for example where 0·2 c.c. of calcium chloride solution had been employed, partial haemolysis usually occurred. A control series of tubes were set up in which Calgon was added to serum heated to 55° C. for ½ hour to destroy complement and then calcium chloride solution added as described above. The addition of calcium chloride caused similar precipitates, but after centrifuging, 0·1 c.c. quantities tested as before showed no haemolysis, indicating that the calcium chloride itself was not haemolytic.

From these experiments it is clear that the addition of calcium chloride precipitates the excess of hexametaphosphate. The restoration of complement may be due to either:

(*a*) Replacement of calcium ions previously removed by hexametaphosphate, or—

(*b*) The removal of hexametaphosphate combined with the serum proteins.

It will also be noted that the concentrations of calcium chloride employed were not high enough to bring about the removal of complement activity as noted by Wright and MacCallum (1922).

In view of the possibilities outlined above, a third series of experiments was carried out to see if calcium ions were necessary for complement action. To three quantities of 2 c.c. guinea-pig serum were added 2 c.c. normal saline and (*a*) 0·25, (*b*) 0·5, and (*c*) 1·0 c.c. of saturated ammonium oxalate solution. The tubes were allowed to stand on the bench for two hours. Calcium oxalate was precipitated; the supernatant liquid was poured off and titrated for complement activity. A control guinea-pig serum untreated with ammonium oxalate was diluted in a similar manner and also tested for complement activity. It was found that the complement titre of all these sera was not diminished by the removal of calcium ions. Determinations of calcium on the deposits (kindly carried out by Dr. F. S. Fowweather) resulted as follows: (*a*) 7·2 mg., (*b*) 7·5 mg., (*c*) 7·5 mg. Ca per 100 c.c. serum.

Care was observed in using sodium chloride free from calcium salts in diluting the sera for titration purposes.

This set of experiments makes it quite clear that the removal of calcium ions does not interfere with complement activity.

Thus the restoration of complement activity by addition of calcium chloride noted in the second series of experiments is not due to the replacement of calcium ions, and therefore the alternative explanation that calcium chloride is effective by removing the hexametaphosphate from combination with the serum proteins would appear to be more likely. The question now arises as to whether sodium hexametaphosphate functions as a salt causing dispersion of the serum proteins, lyotropic effect, or as a species of tanning agent, chemical combination with protein, as demonstrated by Wilson (1937).

TABLE I.

No. of tube.	Serum used. (c.c.)	Result.	No. of tube.	Serum used. (c.c.)	Result.
1	0·1 A	3	40	0·1 P + 0·1 F	0
2	0·1 A + 0·1 heated	4	41	0·1 P + 0·1 L	4
3	0·1 A + 0·1 2% egg	4	42	0·1 Q + 0·1 B	3
4	0·1 A + 0·9 saline	4	43	0·1 Q + 0·1 F	0
5	0·1 B	0	44	0·1 Q + 0·1 L	4
6	0·1 B + 0·1 heated	3	45	0·1 R + 0·1 B	2
7	0·1 B + 0·1 2% egg	0	46	0·1 R + 0·1 F	0
8	0·1 B + 0·9 saline	0	47	0·1 R + 0·1 L	3
9	0·1 C	0	48	0·1 S + 0·1 B	2
10	0·1 C + 0·1 heated	0	49	0·1 S + 0·1 F	0
11	0·1 C + 0·1 2% egg	0	50	0·1 S + 0·1 L	3
12	0·1 C + 0·9 saline	0	51	0·1 P + 0·1 C	0
13	0·1 D	0	52	0·1 P + 0·1 G	0
14	0·1 D + 0·1 heated	0	53	0·1 P + 0·1 M	4
15	0·1 D + 0·1 2% egg	0	54	0·1 Q + 0·1 C	0
16	0·1 D + 0·9 saline	0	55	0·1 Q + 0·1 G	0
17	0·1 heated serum	0	56	0·1 Q + 0·1 M	4
18	0·1 2% egg albumin	0	57	0·1 R + 0·1 C	0
19	0·1 P	3	58	0·1 R + 0·1 G	0
20	0·1 P + 0·1 heated	4	59	0·1 R + 0·1 M	2
21	0·1 P + 0·1 A	4	60	0·1 S + 0·1 C	0
22	0·1 P + 0·1 E	0	61	0·1 S + 0·1 G	0
23	0·1 P + 0·1 K	4	62	0·1 S + 0·1 M	2
24	0·1 Q	0	63	0·1 P + 0·1 D	0
25	0·1 Q + 0·1 heated	4	64	0·1 P + 0·1 H	0
26	0·1 Q + 0·1 A	4	65	0·1 P + 0·1 N	4
27	0·1 Q + 0·1 E	0	66	0·1 Q + 0·1 D	0
28	0·1 Q + 0·1 K	4	67	0·1 Q + 0·1 H	0
29	0·1 R	0	68	0·1 Q + 0·1 N	4
30	0·1 R + 0·1 heated	4	69	0·1 R + 0·1 D	0
31	0·1 R + 0·1 A	4	70	0·1 R + 0·1 H	0
32	0·1 R + 0·1 E	0	71	0·1 R + 0·1 N	3
33	0·1 R + 0·1 K	3	72	0·1 S + 0·1 D	0
34	0·1 S	0	73	0·1 S + 0·1 H	0
35	0·1 S + 0·1 heated	4	74	0·1 S + 0·1 N	2
36	0·1 S + 0·1 A	4	75	0·1 K	0
37	0·1 S + 0·1 E	0	76	0·1 L	0
38	0·1 S + 0·1 K	2	77	0·1 M	0
39	0·1 P + 0·1 B	3	78	0·1 N	0

Effect of Dilution.

Gordon and Thompson (1933) have shown that additions of salts have the effect of removing complement activity, and the order of the degree of effectiveness follows the Hofmeister series of ions. Dilution of the various salt solutions, however, led to the restoration of haemolytic complement, showing that the action of the salts was not irreversible.

During the course of this investigation a large number of experiments have been carried out and most of the results have been collected in Table I. In Table I the following sets of tubes demonstrate that dilution with normal saline of sera containing hexametaphosphate in concentrations which are sufficient to inactivate complement does not bring about a restoration: Nos. 5 and 8; Nos. 9 and 12; Nos. 13 and 16. It will be noted that the original concentration of hexametaphosphate has been reduced to one-tenth without restoration of complement activity. Had the effect of hexametaphosphate been simply of a lyotropic nature such a degree of dilution would have restored the complement activity. The conclusion must therefore be drawn that there is chemical combination of hexametaphosphate with the serum proteins.

There appears to be a similarity between the actions of hexametaphosphate and Congo Red in inactivating complement. Each can have its action reversed, the former by addition of calcium chloride and the latter is removed by adsorption on charcoal (Gordon, 1930). Neither is affected by dilution.

KEY TO TABLE.

A, B, C, D: These represent amounts of 5 per cent. Calgon solution added to 2 c.c. of normal guinea-pig serum:

A, 0·2 c.c.; B, 0·3 c.c.; C, 0·4 c.c.; D, 0·5 c.c.

E, F, G, H: These represent amounts of 5 per cent. Calgon solution added to 2 c.c. of normal guinea-pig serum which had been previously heated to 55° C. for ½ hour and then cooled.

E, 0·2 c.c.; F, 0·3 c.c.; G, 0·4 c.c.; H, 0·5 c.c.

K, L, M, N: These represent amounts of 5 per cent. Calgon solution added to 2 c.c. of normal guinea-pig serum previously heated as described above. Quantities of 1·6 c.c. of these solutions were then treated with 0·6 c.c. of 2½ per cent. calcium chloride solution and centrifuged to remove the deposit.

K, 0·2 c.c.; L, 0·3 c.c.; M, 0·4 c.c.; N, 0·5 c.c.

P, Q, R, S: These represent amounts of N/1 ammonia added to 4 c.c. of normal guinea-pig serum, incubated for 1½ hours at 37° C. and then equivalent amounts of N/1 hydrochloric acid added to bring the reaction to pH values between 7·0 and 7·3.

P, 0·2 c.c.; Q, 0·3 c.c.; R, 0·4 c.c.; S, 0·5 c.c.

"heated" in the table means heated serum as described above.

"2% egg" means a 2 per cent. solution of egg albumin.

"saline" means normal saline, that is 0·85 per cent. solution of pure sodium chloride.

The numbers in the results column relate to the degree of haemolysis when 0·5 c.c. of a 4 per cent. suspension of sensitized ox-blood cells have been added to the other contents of the tubes and incubated at 37° C. for 2 hours.

4 is complete haemolysis.
3 is nearly complete haemolysis.
2 is partial haemolysis.
1 is trace of haemolysis.
0 is no haemolysis.

It will be noted in Table I that the addition of heated serum restores complement activity to serum previously inactivated by sodium hexametaphosphate (tubes Nos. 5 and 6). This is similar to the effect produced on Congo Red inactivated serum, where it was assumed that on the addition of heated serum a redistribution of the dyestuff occurred whereby the Congo Red was partially removed from the original serum and transferred to the heated serum. The reversal of Congo Red could also be effected by addition of egg albumin solution, but apparently albumin does not absorb hexametaphosphate to any material extent (tubes Nos. 7, 11, 15). There is, however, a slight indication of such adsorption as is shown in tubes Nos. 1 and 3. On the other hand, in tubes Nos. 9 and 10, addition of heated serum does not restore complement activity previously removed by the addition of sodium hexametaphosphate. The reason for this behaviour must be that if the concentration of hexametaphosphate exceeds a certain amount, the excess reacts with the proteins of the heated serum and so inhibits their power of restoring haemolytic complement. In previous work the relation between calcium content and the fourth component of complement (Gordon, Whitehead and Wormall, 1926*a*) had been discussed. It is known that ammonia-treated serum and also hexametaphosphate-treated serum are each devoid of complement activity. In ammonia treated serum it is the fourth component that is inactivated. If the fourth component is not affected by hexametaphosphate, mixing the two inactivated sera should bring about a restoration of complement activity. Actually this is so, as may be seen from consideration of the tubes Nos. 5, 24 and 42. Tube No. 5 represents serum inactivated completely with the minimum amount of hexametaphosphate, tube No. 24 is ammonia inactivated serum, and tube No. 42 is a mixture of the contents of the other two tubes. It will be seen that in tube No. 42 complement activity has been restored. In similar sets of tubes (Nos. 9, 24 and 54), where more hexametaphosphate had been employed there was no restoration of complement, indicating that excess of hexametaphosphate over that required to inactivate serum becomes available to react in the mixture, and the conclusion must be drawn that hexametaphosphate does not inactivate fourth component. The results shown in the table demonstrate clearly the importance of threshold or minimal quantities necessary for inactivation and re-activation. Further examples of the quantitative aspect of these experiments are provided in the series of tubes Nos. 5, 24, 42 and 43, to some of which reference has already been made. Tube No. 5 shows the inactivation of complement by the minimal amount of hexametaphosphate, tube No. 24 shows inactivation by ammonia treatment, also in minimal quantity, tube No. 42 shows the restoration of complement by mixture of sera treated respectively with hexametaphosphate and with ammonia. Tube No. 43 shows that serum first inactivated by heat and then treated with hexametaphosphate is no longer capable of restoring complement activity when added to ammonia inactivated serum. In fact from the consideration of tubes Nos. 19 and 22, there is evidence that hexametaphosphate-heated serum is anti-complementary. A suggested explanation for this behaviour is as follows: After heat treatment the serum proteins are not as reactive to hexametaphosphate as before. Thus there is a larger amount of free hexametaphosphate which is sufficient to react with the serum proteins

incompletely inactivated by the ammonia, and so prevents the restoration of complement activity. The effect of hexametaphosphate on heat-inactivated serum is not irreversible. It is possible to restore complement activity in ammonia-inactivated serum if calcium chloride be added to heated serum treated with hexametaphosphate. An inspection of the table shows this behaviour in an unmistakable fashion. Actually there are four series of tubes that may be examined. These are: (*a*) Tubes Nos. 23, 28, 33 and 38; (*b*) tubes Nos. 41, 44, 47 and 50; (*c*) tubes Nos. 53, 56, 59 and 62; (*d*) tubes Nos. 65, 68, 71 and 74.

Some of the tubes mentioned above include those where initially large additions of hexametaphosphate have been made. Normally these would have completely removed complement activity, but the addition of calcium chloride has removed the hexametaphosphate from combination with the protein.

EFFECT OF PHOSPHORIC ACIDS.

It was considered desirable to test the effects of the salts of the different phosphoric acids on the inactivation of complement activity. It is well known that only metaphosphates precipitate albumin from acid solution, and they are also the only phosphoric acid derivatives with tanning properties on animal skins. Di-sodium orthophosphate was found to be ineffective in its action on serum complement, but sodium pyrophosphate had a slight action in removing complement, but only in relatively high concentration and was reversible on dilution. It can therefore be fairly concluded that pyrophosphate acts through its comparatively high dispersing effect on the protein aggregates, and in fact it has been used in the leather industry for facilitating the soaking of hard dried skins to a state of softness and suppleness usually encountered with ordinary fresh hides and skins. The softening effect is ascribed to the solution of dried inter-fibrillary proteins which are akin to hard-boiled white of egg.

The following is a brief summary of the results obtained using 5 per cent. solutions of sodium salts of the various phosphoric acids which had been adjusted to pH 7·4. Varying amounts of the different phosphate solutions were added to 1 c.c. of normal guinea-pig serum:

c.c. of 5 per cent. solution.	Type of phosphate.	Complement inactivation.
0·1	Calgon	None.
0·2	,,	Complete.
0·8	Sodium pyrophosphate	Partial.
0·9	,, ,,	Complete.*
1·0	,, orthophosphate	None.

* On dilution with saline complement activity was restored.

ACTION OF HEXAMETAPHOSPHATE ON THE IMMUNE BODY.

There remains the somewhat remote possibility that the inactivation of complement is not due to the reaction between the serum proteins and hexa-

metaphosphate, but is the result of the action of the latter on the immune body absorbed on the red blood cells. In consequence of this possibility quantities of Calgon in amounts previously found to be in excess of that required for inactivation of complement were added to guinea-pig serum. This treated serum produced no haemolytic action on sensitized red blood cells. The latter were then spun down on the centrifuge, supernatant liquid removed and the cells further washed by successive centrifuging operations. Addition of untreated guinea-pig serum now gave complete haemolysis. It is thus clear that hexametaphosphate has no permanent effect on sensitized red blood cells in preventing haemolysis as it has on the proteins of serum.

The tanning action of hexametaphosphates is obviously due to the reaction between the basic groups of the protein and the complex anion of the hexametaphosphate. In order that this reaction should proceed it is necessary for acid to be present, and this has the effect of back-titrating the negatively charged $-COO^-$ parts of the protein "Zwitterions" and converting them into uncharged $-COOH$ groups. The positively charged $-NH_3$ groups of the protein side chains are then free to combine with the hexametaphosphate anions to form the insoluble precipitates with protein solutions, such as those of albumin or the analogous leather when the protein is in a fibrous condition as is the case with hide. In the experiments described in this paper the reactions have taken place at pH 7·4 and the protein "Zwitterions" are intact. Nevertheless a reaction does take place to a certain extent in which the complex anion of the hexametaphosphate must be competing with the protein anions for combination with the positively charged basic groups of the proteins in serum. Apparently this reaction need not be very far-reaching in order to remove the complement activity.

SUMMARY.

Sodium hexametaphosphate (Calgon) when added to serum removes complement activity.

This effect is reversed by the addition of calcium chloride.

Hexametaphosphates remove calcium ions from solution, but the restoration of complement activity is not to be ascribed to the replacement of calcium, but is to be explained by calcium chloride removing hexametaphosphate from combination with the serum proteins.

The inactivation by Calgon is not an ordinary lyotropic salt effect, because dilution does not restore complement activity.

Hexametaphosphates and Congo Red exhibit parallel effects both in the inactivation of complement and in reversibility.

Hexametaphosphates behave differently in their reactions with proteins from salts of other varieties of phosphoric acid. This applies to the action on serum complement and on the tanning of hides and skins.

We should like to express our indebtedness to the Medical Research Council for a grant towards the expenses of this work.

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STUDIES ON THE EFFECT OF CARCINOGENIC HYDROCARBONS ON IMMUNITY REACTIONS.

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From the Chester Beatty Research Institute, the Royal Cancer Hospital (Free), London, S.W. 3.

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In the present experiments the effect of carcinogenic hydrocarbons on the formation and the amount of precipitating antibodies has been investigated.

The experiments are divided into two main lines:

(1) The effect of treatment with carcinogenic hydrocarbons, prior to immunization, on the formation of antibodies.

(2) The effect of carcinogenic agents, applied at the stage of high immunity, on the degree of immunity.

For the estimation of the amount of antibodies generally two methods have been used: the method of optimal proportions of Dean and Webb (1926), and the determination of the amount of nitrogen in the antigen-antibody precipitate. In some of the experiments the antibodies were estimated by the dilution method.

PART I.

Four rabbits received polycyclic hydrocarbons during one year. One animal was treated with methylcholanthrene, one with 1 : 2 : 5 : 6-dibenzanthracene, one with 1 : 2-benzanthracene and one with phenanthrene. The hydrocarbon was injected as a 0·05 per cent. colloid in a 1 per cent. watery gelatine solution. The injections were given subcutaneously, 5 c.c. each time at four to eight weeks' intervals. The animals looked healthy at the beginning of the immunization. As antigen, horse serum was injected intravenously twice weekly during two weeks and once intraperitoneally in the third week, 2 c.c. each time. At the end of the second and third week, and 10 days after

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DIONIZY GORNY

CONTENT OF ADRENALINE AND NORADRENALINE IN THE BRAIN, HEART AND ADRENALS OF RATS AFTER ADMINISTRATION OF SODIUM PHOSPHATE, HYDROCORTISONE AND DEOXYCORTICOSTERONE

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Head: Professor J. Billewicz-Stankiewicz, M. D.

GORNY D.: *Content of Adrenaline and Noradrenaline in the Brain, Heart and Adrenals of Rats after Administration of Sodium Phosphate, Hydrocortisone and Deoxycorticosterone.* Acta Physiol. Pol. 19 (6): 825—834, 1968. — Experiments were performed on 60 white rats. Sodium phosphate, in a dose amounting to 2 mM per 24 hrs, was given through the catheter directly into the stomach. Hydrocortisone and deoxycorticosterone were applied i.m. every day for 12 days in single doses of 1.2 µg per 1 g of body weight. Catecholamines content in organs was fluorometrically determined. Sodium phosphate, when administered during the autumn-winter period, caused a general increase of catecholamines content in brain, heart and adrenal glands. Significant increase of noradrenaline level and decrease of adrenal gland weight by 21.5 percent was stated. Sodium phosphate, when applied in the spring-summer period, did not cause any changes of the catecholamine content nor in the adrenal glands weight. Hydrocortisone when applied in rats receiving phosphate, lossed its property of decreasing the adrenaline content in heart and brain. Adrenal glands revealed, in this case, a small weight increase instead of decrease.

Similarity of acute necrosis of myocardium observed in rats after phosphate and corticosteroid hormones administration [25] with the necrosis observed after the sympathicomimetic amines administration [8, 15, 22, 23, 26], after physical effort [25], or as a result of stress [25] suggests a strong engagement of the sympathetic nervous system and catecholamines in the process.

METHODS

Investigations were performed on male and female albino rats, bastards, weighing 110—220 g. Because of carrying out the experiments in different seasons of the year, the animals were divided into two groups: autumm-winter and spring-summer.

The autumn-winter group was tested for the behavior of catecholamines after administration of only monobasic sodium phosphate as well as after application of phosphate together with hydrocortisone and deoxycorticosterone.

In the spring-summer group the action of the phosphate, than the action of the phosphate along with hydrocortisone or deoxycorticosterone as well as the action of phosphate together with both hormones was examined.

Each of the six groups of animals contained 10 rats at the beginning of experiments. In the meantime about 25 percent of the rats fell sick and therefore the number of animals was changed (Tables 1, 2 and 3). The cause of the illness most often were the inflammatory-necrotic changes of myocardium.

The hormones (firm-Polfa) were injected intramuscularly every day for 12 days in the doses of 1.2 µg per 1 g of body weight. The monobasic sodium phosphate was given through the catheter directly into the stomach, in two daily doses, amounting 5 ml each, which corresponded to 2 mM pure substance per 24 hrs. The control animals were given 0,25 ml of 0.9 percent NaCl intramuscularly (control for hydrocortisone) and 0.25 ml of vegetable oil (control for deoxycorticosterone) and water by means of the stomach catheter (control for phosphate). After 12 days the animals were drugged with ethyl chloride and killed by heart blood aspiration. The brain, heart and suprarenal gland were removed, weighed and homogenized in 5 percent solution of trichloroacetic acid with sodium versenate and ascorbic acid. The extraction time amounted to 30—45 minutes and the temperature about 4°C. The homogenized tissue was centrifuged. Catecholamines in the supernatant fluid were adsorbed by means of alumina, rinsed by 1 N acetic acid and then determined fluorometrically [4]

RESULTS

Autumn-winter group

After application of monobasic sodium phosphate, there appeared a statistically significant increase of noradrenaline content in the brain, heart and suprarenal gland and an insignificant increase of adrenaline in these organs. The weight of the suprarenal glands decreased (Table 1, 2 and 3).

Both steroid hormones connected with phosphate revealed no influence on the content of catecholamines in the organs under investigation. It was stated only a change in the weight of suprarenal gland. They weighed 36 percent less in comparison to the glands of the parallel control group (Table 1, 2 and 3).

Spring-summer group

Sodium phosphate did not change the concentration of catecholamines in the tested organs and the weight of suprarenal glands of the experimental animals was the same as in the control group (Table 1, 2, and 3).

Sodium phosphate when given with hydrocortisone did not change the concentration of catecholamines in the rat's brain, heart and suprarenal glands. The suprarenal glands weighed by 10.7 percent more than the glands derived from control animals preserving almost the same body weight (Table 1, 2 and 3).

Table 1. Number of animals, mean body weight and mean weight of brain as well as mean concentration of adrenaline and noradrenaline in rat brain after administration of sodium phosphate, hydrocortisone and deoxycorticosterone

	Autumn-winter period			Spring-summer period				
	Control	NaH_2PO_4	NaH_2PO_4 DOC HCS	NaH_2PO_4	NaH_2PO_4 DOC HCS	NaH_2PO_4 HCS	NaH_2PO_4 DOC	Control
Number of animals	22	9	8	7	8	8	8	7
Weight of animals	232	171	195	171	181	187	215	196
Weight of brain, mg	1645	1636	1690	1659	1618	1678	1684	1606
Adrenaline, μg/g	0·0538 ±0·004517	0·063 ±0·00577	0·0507 ±0·00969	0·0408 ±0·00963	0·0503 ±0·00897	0·0356 ±0·00302	0·0334 ±0·00571	0·0383 ±0·00589
P		<0·3	<0·8	<0·9	<0·3	<0·7	<0·6	
Adrenaline, percent	9·4	8·46	7·94	7·27	9·98	7·65	6·21	7·9
Noradrenaline, μg/g	0·5184 ±0·02984	0·682 ±0·03675	0·5875 ±0·04768	0·5207 ±0·03777	0·4538 ±0·02499	0·4298 ±0·04006	0·5041 ±0·05475	0·5021 ±0·02089
P		<0·01	<0·3	<0·7	<0·2	<0·2	<0·9	
Noradrenaline, percent	90·60	91·54	92·06	92·10	90·02	92·35	93·79	92·10

Table 2. Number of animals, mean body weight and mean weight of heart as well as mean concentration of adrenaline and noradrenaline in rat heart after administration of sodium phosphate, hydrocortisone and deoxycorticosterone

	Autumn-winter period			Spring-summer period				
	Control	NaH_2PO_4	NaH_2PO_4 DOC HCS	NaH_2PO_4	NaH_2PO_4 DOC HCS	NaH_2PO_4 HCS	NaH_2PO_4 DOC	Control
Number of animals	18	9	8	7	8	8	8	7
Weight of animals	211	171	195	175	181	187	215	196
Weight of heart, mg	854	671	753	741	756	670	698	840
Adrenaline, μg/g	0·126 ±0·0159	0·156 ±0·01418	0·1145 ±0·1141	0·0701 ±0·00901	0·0537 ±0·01241	0·0814 ±0·01275	0·0795 ±0·01457	0·0687 ±0·0111
P		<0·2	<0·6	<0·9	<0·4	<0·5	<0·6	
Adrenaline, percent	14·52	10·03	10·51	6·87	5·09	8·82	9·06	7·6
Noradrenaline, μg/g	0·8550 ±0·07231	1·3990 ±0·279	0·9752 ±0·06799	0·9489 ±0·1042	1·0018 ±0·06311	0·8415 ±0·04734	0·7984 ±0·07908	0·8349 ±0·1435
P		<0·01	<0·3	<0·6	<0·3	<0·9	<0·9	
Noradrenaline, percent	85·48	89·97	89·49	93·13	94·91	91·18	90·94	92·40

Table 3. Number of animals, mean body weight and mean weight of suprarenal glands as well as mean concentration of adrenaline and noradrenaline in rat suprarenal glands after administration of sodium phosphate, hydrocortisone and deoxycorticosterone

	Autumn-winter period				Spring-summer period				
	Control	NaH_2PO_4	NaH_2PO_4 HCS DOC	NaH_2PO_4	NaH_2PO_4 HCS DOC	NaH_2PO_4 HCS	NaH_2PO_4 DOC	Control
Number of animals	10	9	8	7	8	9	8	6
Weight of animals	184	171	195	174	181	184	215	191
Weight of suprarenal glands, mg	42·30	33·22	27·13	41·30	36·62	46·56	44·0	42·05
Adrenaline, μg/2 glands	28·33 ±4·47	34·58 ±1·377	30·89 ±1·393	35·90 ±1·910	31·25 ±1·143	36·88 ±4·211	41·48 ±3·409	35·85 ±4·30
P		<0·2	<0·6	<0·9	<0·4	<0·9	<0·4	
Adrenaline, percent	81·96	76·10	82·81	82·76	81·08	80·77	82·70	86·50
Noradrenaline, μg/2 glands	6·24 ±0·8003	10·86 ±1·303	6·41 ±0·4044	7·48 ±0·5667	7·30 ±0·5898	8·78 ±1·699	8·68 ±0·8847	5·62 ±0·8737
P		<0·01	<0·9	<0·2	<0·2	<0·2	<0·05	
Noradrenaline, percent	18·04	23·90	17·19	17·24	18·94	19·23	17·30	13·50

Sodium phosphate together with deoxycorticosterone raised significantly the noradrenaline content in suprarenal glands, not changing the content of adrenaline and the two catecholamines in heart and brain (Table 1, 2 and 3). The weight of suprarenal glands as related to the body weight was the same as for the glands of the control group (Table 3).

Simultaneous application of phosphate connected with the two steroid hormones did not change the catecholamines content in the investigated organs (Table 1, 2 and 3). The suprarenal glands were by 13 percent lighter than the glands of the corresponding control group (Table 3).

DISCUSSION

Catecholamines, corticosteroids and inorganic phosphate compounds reveale in the living organism a rather intimate mutual functional dependency.

As it results from bibliographical data the application of labelled phosphorus causes a threefold increase of corticosterone in blood plasma of mice, and the circadian fluctuation in the serum phosphorus level coincides with changes of corticosteroids secretion [20]. Glicocorticoids have been found to reduce renal tubular reabsorption of inorganic phosphate [3, 13], increase the adenosine phosphate (ATP) content as well as the phosphatase activity [21] in the liver. *Arvay et al.* showed in rats, that stressing agents like light, noise or stimulation with electric current increased the secretory function of the suprarenal cortex. As associated symptoms were found: the increase of radio-active phosphorus accumulation and of phosphatase activity in suprarenal glands. *Hevesy* and *Santo* showed in mice and rabbits that adrenaline increases the transfer of phosphorus from blood to the tissues and reduces by 20 percent the accumulation of phosphorus by bones, but does not change its penetration into the liver tissue. *Pincus* and *Cori* observed after adrenaline application similar changes in blood. *Kenny* obtained in rats, after adrenaline and isoproterenol (0·01—5 mg) a 50 percent decrease of phosphorus content in blood but did not observe any changes in the content of calcium. Noradrenaline did not show any activity in these conditions. In animals deprived of parathyroids all of the three amines caused a decrease of phosphorus in blood and an increase in calcium content. The blocking of beta receptors with nethalide abolished the catechol amines activity for the phosphorus transfer from blood to tissues. As results from investigations of *Morey* and *Kenny* noradrenaline increases the excretion of phosphorus in rat urine. The activity disappears after parathyreoidectomy. The effect of adrenaline depended on the dose applied as well as on preserving the parathyroid gland. Adrenaline, contrary to noradrenaline, administered in

doses of 0·1 mg per kg decreases the phosphorus content in urine, and after parathyreoidectomy it caused hyperphosphaturia.

Hypophosphatemia, as affected by adrenaline, is probably the result of an increase of glicolysis in tissues. It was confirmed in the course of investigations on cell cultures as well as on isolated organs or in the whole organism. Glicolysis reduces the content of inorganic phosphorus in cells of the Erlich tumor, increases its collection from the environment and incorporation into organic fractions with formation of hexosophosphates and ATP (10, 11).

Intravenous infusion of adrenaline increases in man the lactic acid and pyruvic acid content and decreases the phosphorus level in the venous blood (12). As results from *Lundholm's* and *Mohme-Lundholm's* investigations adrenaline with glucose in anaerobic conditions increase the lactic acid content in the environment surrounding the beaf intestine. They [illegible] ation of ATP, ADP and creatine phosphate, decreasing [illegible] the inorganic phosphate concentration in the corresponding vascular system. Adrenaline acts analogically onto passively stretched intestinal smooth muscles of guinea pig [2], and on an isolated rat's diaphragm [6]. On the other hand inorganic phosphates increase the glicolytic activity of adrenaline [26] and increase glucose level in blood [19].

It is allready known that inorganic phosphate is indispensable in the course of anaerobic glicolysis. It participates in the process of oxidation of 3-phospho-gliceraldehyde. The depletion of inorganic phosphate and the accumulation of triosophosphates in cells disturbes energetic relationship of the reaction catalysed by aldolase and triosophosphate isomerase, and therefore glicolysis stops at the hexosophosphate stage [10, 11]. The stimulation of glicolysis by phosphorus progresses not only in yeast cells but also in Ehrlich tumor cells (10, 11, 31), in white and red blood cells and thrombocytes of man and experimental animals (16, 17, 27, 30) as well as in striated muscles [28], and in the liver [29].

On the basis of carbohydrate metabolic analysis in the Ehrlich tumor cells it was found that inorganic phosphorus compounds activate hexokinase, phosphofructokinase and dehydrogenase of 3-phospho-glyceraldehyde [29, 31]. These enzymes are interdependent, and the primary activation of phosphofructokinase supplies the fructose-diphosphate and triosophosphate being the substrate for the dehydrogenase 3-phospho-glyceraldehyde. Simultaneously decreases the concentration of hexoso-1-phosphate in the cell and the action of the hexokinase is increased. Similar results were obtained by *Minakami* and *Yoshikawa*, and *Tsuboi* and *Fukuoka* in the course of investigations carried out on human erythrocytes.

The results obtained in the present study are indicating that the effect of sodium phosphate on the content of catecholamines in the tested organs, as well as on the size of suprarenal glands is different in the autumn-winter period than in the spring-summer one. During the autumn-winter period the phosphate caused a general rise of catecholamine content in tested organs together with a significant increase of noradrenaline and by 21·5 percent decrease of suprarenal gland weight. Any changes in the content of catecholamines, and in the weight of the suprarenal glands during the spring-summer period were not stated. It may be supposed that phosphate activity depends of the initial background, i.e. on the biological state of the tissiues which as results from personal investigations, is changing in various seasons. Moreover, as it was found by us previously [5] hydrocortisone applied with phosphate losses its ability to decrease the adrenaline content in heart and brain of rats. Under such conditions, the suprarenal glands react instead of decresing by increasing their weight. Deoxycorticosterone, when applied with phosphate, increases the content of noradrenaline in suprarenal glands, what was not found after applying deoxycorticosterone alone [5].

The results obtained confirm the functional relation between adrenocorticosteroids, catecholamines and sodium phosphate, but their biochemical mechanisms cannot be at present explained on the basis of the evailable literature. Other nearly unknown biochemical processes occurring in the body should be taken into consideration.

CONCLUSIONS

1. Sodium phosphate administered to animals during the autumn-winter period, caused a general increase of catecholamine content in brain, heart and suprarenal gland tissue. The significant increase of noradrenaline content and decrease of suprarenal gland weight by 21·5 percent was stated.

2. Sodium phosphate, when applied in the spring-summer period, did not cause any changes of the catecholamine content in the examined organs nor in the weight of suprarenal glands.

3. Simultaneous application of sodium phosphate with hydrocortisone in spring-summer period did not change the catecholamine level in the examined organs. The suprarenal glands showed a weight increase by 10·7 percent in comparison to the glands of control animals.

4. Deoxycorticosterone, applied together with phosphate, increased significantly the noradrenaline content in suprarenal glands, without changing the adrenaline and both catecholamines level in the brain and heart.

5. Both steroids, connected with phosphate, did not reveale any effect on the catecholamine content in examined organs of autumn-winter and spring-summer group of rats. The weight of suprarenal glands of au-

tumn-winter and spring-summer group was decreased by 36 percent and 13 percent, respectively, in comparison to the weight of glands of parallel control group.

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METABOLIC ACIDOSIS AND HYPOCALCEMIA AS TOXIC MANIFESTATIONS OF POLYMERIC PHOSPHATES[1]

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Pyro- and metaphosphates have long been thought to be more injurious than orthophosphates although none can be regarded as highly toxic (Gambee, Priestley and Larmuth, 1877). Starkenstein (1911) emphasized an "alkaliwirkung" as the manner by which Na_3PO_4 and $Na_4P_2O_7$ produce death, but he later suggested (1914) that the calcium-binding properties of sodium phosphate polymers might explain why they are more toxic than orthophosphate salts. Behrens and Seelkopf (1932) and Jones and Murray (1937) demonstrated conclusively that untoward reactions may result from reductions in the plasma level of ionized calcium because calcium forms complex-ions with phosphate polymers *in vivo*.

In this laboratory, recent studies of the metabolism of several inorganic condensed phosphates (Gosselin *et al.*, 1952) demonstrated that their hydrolysis led to a severe systemic acidosis. In fact, with the exception of the sequelae of direct intravenous administration of the polymer solutions, acidosis was generally a more prominent phenomenon than hypocalcemia. The following data concern the acute toxicity of several phosphate polymers when given parenterally to rats; of particular interest is the soluble phosphate glass available commercially as sodium hexametaphosphate. An attempt is made to evaluate the relative importance of acidosis and of hypocalcemia in these observations.

MATERIALS AND METHODS: Commercial sources and the chemical nature of phosphates used in this study have been summarized previously (Gosselin *et al.*, 1952). These compounds can be represented as polymers of orthophosphate with the following molecular formulas of the sodium salts: trimetaphosphate (a cyclic trimer) $Na_3P_3O_9$; tetrametaphosphate (a cyclic tetramer) $Na_4P_4O_{12}$; pyrophosphate (a linear dimer) $Na_4P_2O_7$; tripolyphosphate (a linear trimer) $Na_5P_3O_{10}$; and hexametaphosphate (a mixture of linear polymers with 6.0 atoms of P in the average molecule) $Na_3P_6O_{19}$. By replacing some of the sodium with calcium, salts of hexametaphosphate (HMP) were prepared with various Ca/P mol ratios. Except as noted, all solutions and suspensions were adjusted to pH 7.4 (glass electrode) before injection. All doses are expressed in terms of phosphorus present as labile[4] phosphate.

Rats in all-glass metabolism cages furnished 24-hour urine specimens with minimal fecal contamination. Dilute aliquots of urine were titrated to pH 7.4 with 0.01 *N* NaOH.

[1] This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, N. Y.

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[4] Orthophosphate released by hydrolysis in 1.0 *N* HCl during seven minutes at 100°C.

Phos

Urinary ammonia analyses were based on direct nesslerization after the removal of interfering substances by the method of Sumner (1918). Arterial plasma levels of CO_2 were measured manometrically by the method of Van Slyke and Neilson. Each rat was lightly anesthetized with ether or with ether and intraperitoneal pentobarbital, and a single blood sample was obtained from the abdominal aorta. These samples were treated with both heparin and NaF. Plasma was obtained anaerobically by centrifugation within the collecting syringe equipped with an adapter of the type described by Davenport (1950). Electrocardiograms on pentobarbitalized rats were recorded from standard limb leads using subcutaneous needle electrodes, a condenser-coupled amplifier, and a pen-writing oscillograph.

Intraperitoneal toxicity: In table 1, sodium hexametaphosphate (Na-HMP) is compared with other condensed phosphates in respect to the LD_{50} doses when these substances were given to rats by single intraperitoneal injection. It is apparent that Na-HMP is intermediate between the relatively toxic pyrophosphate and the nearly innocuous cyclic polymers trimeta- and tetrameta-

TABLE 1

*Acute toxicity of phosphates in rats**

Intraperitoneal LD_{50} (24-hour mortality)†

COMPOUND	NO. RATS	BODY WT.	SEX	LD_{50}	
				mgm. salt/kgm.	mgm. P/kgm.
		gm.			
a. Na pyrophosphate	68	171–245	F	243 ± 13	89
b. Na tripolyphosphate	74	160–254	F	525 ± 52	135
c. Na hexametaphosphate	29	252–404	M	690 ± 160	192
d. Na tetrametaphosphate	113	151–263	M	2550 ± 250	741
e. Na trimetaphosphate	57	148–250	F	3650 ± 620	1130
f. Na orthophosphate‡	70	172–305	M		326

* Rats of the Rochester strain (ex-Wistar).

† Calculated by the graphical method of Miller and Tainter (1944).

‡ Mixture of NaH_2PO_4 - Na_2HPO_4 (Solution 1 *M* in P and pH 7.4). Other solutions were not adjusted to pH 7.4.

phosphate. Partial replacement of sodium by calcium markedly lessened the acute toxicity: the 24-hour LD_{50} of Na-HMP proved to be 192 mgm. P/kgm.; that of Ca-HMP (Ca/P mol ratio 0.5) was at least three times greater. Although most fatalities occurred within one to four hours after an intraperitoneal dose of Na-HMP, deaths were often noted several days after large doses of Ca-HMP. Even in sublethal amounts (100 mgm. P/kgm.), all injections of Na-HMP provoked immediate excitement, and in the ensuing days loss of body weight was often severe. Neither pain nor anorexia was obvious after similar doses of Ca-HMP.

Acidosis: Attention has been drawn to the enzymatic hydrolysis of certain phosphate polymers in rats and rabbits (Gosselin *et al.*, 1952). The more toxic compounds in table 1 (a, b, c) are known to be hydrolyzed *in vivo*, releasing unneutralized orthophosphoric acid residues; the others are only slightly hydrolyzed, if at all. As demonstrated in figure 1 by the difference between the titration

curves of orthophosphoric and hexametaphosphoric acids at pH 7.4, the hydrolysis of Na-HMP in solutions at pH 7.4 liberates 0.62 mols H^+ per mol P. In terms of mols H^+ per mol P, the following values were similarly obtained with other sodium polymers: pyrophosphate 0.28, tripolyphosphate 0.54, trimetaphosphate 0.84, and tetrametaphosphate 0.84.

In vivo the hydrolysis of Na-HMP led to a severe metabolic acidosis. In rats this acidosis has been characterized in two ways: by observation of the CO_2 content in arterial plasma and by measurement of fixed acid excretion in the urine. The latter studies are summarized in table 2, which includes mean values of titratable acidity and ammonia content of 24-hour urine samples during two

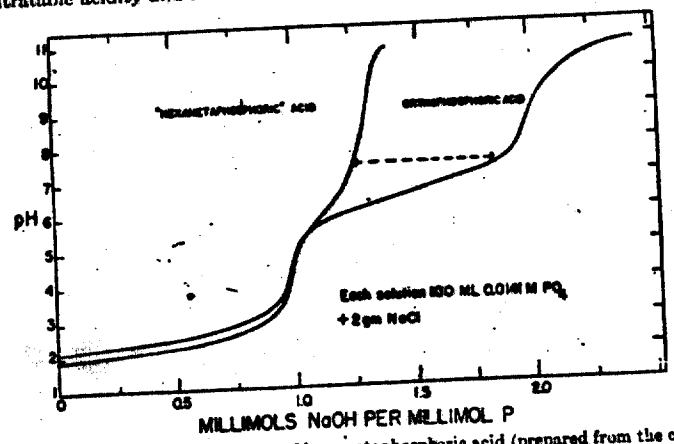


FIG. 1. The electrometric titration of hexametaphosphoric acid (prepared from the commercial sodium salt by a cation-exchange resin) and of orthophosphoric acid (prepared from the polymeric acid by thermal hydrolysis), using 0.100 N NaOH. Sodium chloride was added merely to steepen the curves at their inflection points. A blank obtained by titrating distilled water has been subtracted from each curve. The titration of H-HMP has been corrected for the small amount of orthophosphate present as a contaminant. In an equivalent concentration the commercial sodium salt had a pH of 6.6.

days of fasting. Because some bacterial contamination of these urine samples was inevitable, the ammonia values may have been higher and the titratable acidities correspondingly lower than in freshly voided urine; the sum of two, however, is regarded as a valid measure of the urinary excretion of fixed acid (in excess of fixed base). Rats treated with Na-HMP invariably excreted more acid than their controls during the first day (mean difference of 0.42 meq. H^+); by the second day acid excretion had decreased somewhat below that of the control group.

Although fixed acid excretion during the first day was only 41 per cent higher in treated rats than in controls, the speed at which this acid was produced *in vivo* resulted in a moderately severe systemic acidosis within an hour. Hyperpnea

was usually obvious within a few minutes. Figure 2 shows the arterial plasma content of CO_2 in untreated rats, in those given LD_{50} doses of Na-HMP i.p., and in those given the same amount of phosphorus as Ca-HMP. The mean value of plasma CO_2 in eight control rats was 29.5 meq./l. A prompt and severe reduction in the CO_2 content is apparent in rats treated with Na-HMP. The calcium salt, which is mobilized from the site of injection much more slowly (Gosselin *et al.*, 1952), caused a less marked disturbance in the blood buffers. Since many rats died one to three hours after this dose of Na-HMP, the data in figure 2 are biased in favor of the survivors, and consequently the observed values of CO_2 may well be somewhat higher than those which were reached just

TABLE 2

The effect of Na-HMP administration on the daily urinary excretion of fixed acid by fasting rats

(All values in milli-equivalents)

	CONTROL RATS* (MEAN ± S.E.)	EXPT. RATS* (MEAN ± S. E.)
First 24-hours		
Titratable acidity	0.53	0.60
Ammonia	0.48	0.84
Total fixed acid	1.02 ± 0.06	1.44 ± 0.12
Second 24-hours		
Titratable acidity	0.50	0.36
Ammonia	0.69	0.71
Total fixed acid	1.19 ± 0.02	1.07 ± 0.08
48-hrs.		
Total fixed acid	2.20 ± 0.09	2.51 ± 0.09

* Each value represents the mean for four male rats with body weights between 280 and 300 gm. Each rat of the experimental group received intraperitoneally 29 mgm. P as Na-HMP (pH = 7.4) at the beginning of the fast. Water was provided *ad libitum*.

before death. These data demonstrate that acidosis is one factor which must contribute to the lethality of HMP.

Hypocalcemia: Another factor that contributes to the acute toxicity of HMP is a reduction in the plasma level of ionized calcium by the chelation of Ca^{++} by HMP in the blood stream. Because this type of hypocalcemia is not readily detected by chemical procedures, a biological index was sought. One of the more sensitive indices is a prolongation of the Q-T interval in the electrocardiogram (Ernestene and Proudfit, 1949). Figure 3 illustrates changes in Q-T relative to the cycle length (R-R) in a rat receiving Na-HMP by intravenous infusion. There was a prompt ECG change consistently observed: a progressive lengthening of Q-T. In rabbits a similar phenomenon often resulted in partial fusion of the T-wave with the subsequent P-wave. As seen in figure 3, the Q-T could be returned

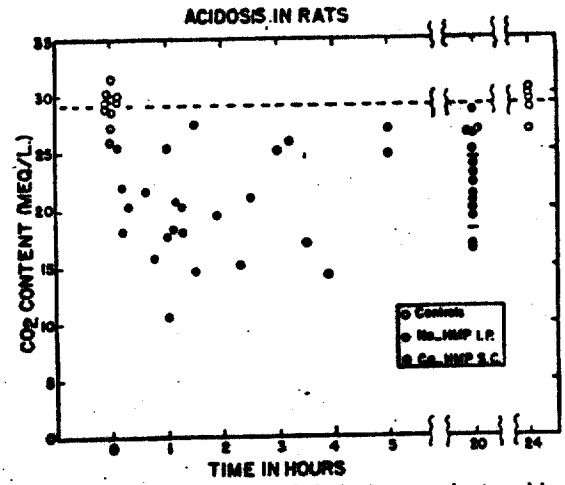
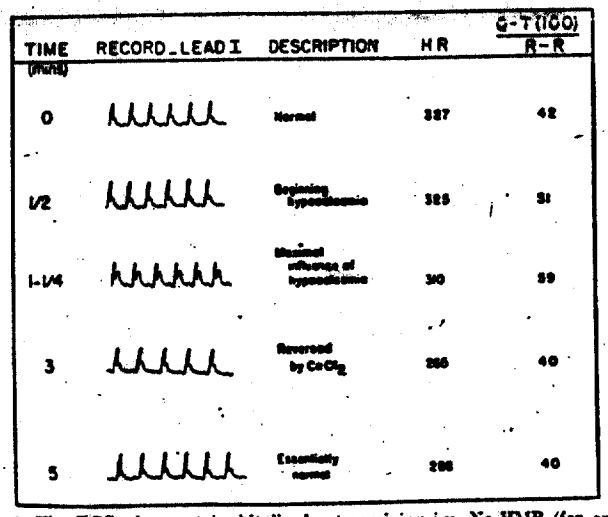


FIG. 2. The arterial plasma content of CO_2 in fasting control rats and in those which received at zero time 100 mgm. P/kgm. as sodium hexametaphosphate (i.p.) or as calcium hexametaphosphate (s.c.).

EEG CHANGES IN A RAT



TIME (Hrs)	RECORD_LEAD I	DESCRIPTION	HR	Q-T(100)/R-R
0		Normal	327	42
1/2		Beginning hypocalcemia	325	31
1-1/4		Maximal influence of hypocalcemia	310	39
3		Reversed by $CaCl_2$	286	40
5		Essentially normal	288	40

FIG. 3. The ECG of a pentobarbitalized rat receiving i.v. Na-HMP (for one min.), followed by i.v. $CaCl_2$ (for two min.). The recorded heart rates (HR) and ratios between Q-T and R-R were calculated from these ECG tracings.

almost as promptly to its normal duration by the infusion of $CaCl_2$, if begun before the appearance of a severe arrhythmia.

Figure 4 is a record of average changes in Q-T (relative to cycle length) in groups of pentobarbitalized rats which received various phosphate polymers by continuous infusion (femoral vein); in all instances the rate of injection was 6.2 mgm. P/min. Because the Q-T interval is also recognized to be a function of the cycle length (Bazett, 1920), and because the precise relationship between the

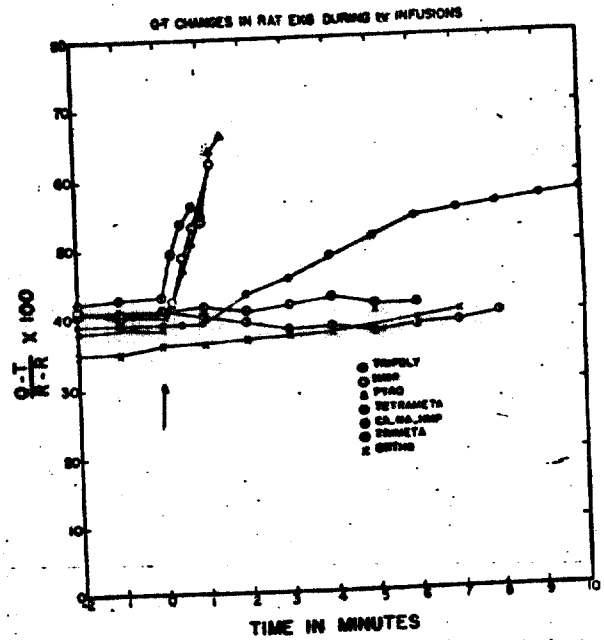


Fig. 4. Average changes in Q-T (relative to R-R) in pentobarbitalized rats which received various phosphate polymers by continuous intravenous infusion (femoral vein); the rate of injection was constant at 6.2 mgm. P/min. All solutions were made from the sodium salts (except for Ca-Na-HMP) and were adjusted to a pH of 7.4. Each point is the mean of at least four rats.

two is unknown in normal rats, the Q-T changes depicted in figures 4 and 5 include only those seen while the heart rate remained at approximately the pre-injection rate (within 15 per cent thereof as an arbitrary limit). The appearance of bradycardia, auriculo-ventricular block, or an arrhythmia invariably terminated the analysis at a time when the Q-T occupied about 60 per cent of the cardiac cycle; cardiac arrest occurred shortly thereafter. This sequence occurred promptly during infusions with sodium salts of HMP, pyro-, and tripolyphos-

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phate. Because larger amounts were required, the prolongation of Q-T was more gradual with Ca-Na-HMP (a clear solution of Na-HMP containing sufficient $CaCl_2$ to yield a Ca/P mol ratio of 0.30). At this rate of infusion, however, sodium salts of ortho-, trimeta-, and tetrametaphosphate had little or no effect on Q-T or heart rate.

As implied in figure 4, HMP proved more toxic than Ca-Na-HMP by the intravenous route, *viz.*, death occurred when the average cumulative dose of HMP reached 48 (±2) mgm. P/kgm. (6 rats), while the lethal dose of Ca-Na-HMP was three to seven times greater at this rate of infusion. Although ECG evidence of a reduction in the plasma level of free calcium was seen in both groups of rats (figure 4), the pattern of response was otherwise markedly different. With Na-HMP, the respiratory rate promptly slowed, but the apical cardiac impulse stopped before or simultaneously with the cessation of breathing. With Ca-Na-HMP respirations were accelerated, cyanosis appeared, frank pulmonary edema was not unusual, and cardiac action invariably outlasted breathing movements. These observations imply that the mode of death was different in the two groups.

The intraperitoneal route of administration revealed further differences in the hypocalcemic activity of these various polymers. Single i.p. doses of Na-HMP produced no ECG evidence of hypocalcemia, even in doses as high as 250 mgm. P/kgm. (4 rats). Similarly with LD_{50} doses of sodium tripolyphosphate, only a small and equivocal upward trend in QT/R-R was observed over a period of three hours (figure 5). In contrast sodium pyrophosphate led to prompt and impressive widening of Q-T, even in the relatively low dose of 50 mgm. P/kgm. (figure 5). Moreover, in those rats which survived this LD_{50} dose, the Q-T began to return toward normal after two hours; in those which eventually died, the Q-T continued to lengthen for at least three hours, when the analysis had to be terminated because of bradycardia and occasionally because of an arrhythmia.

The stability of calcium complexes in vitro: As measured by the mol ratio of Ca to P in precipitates which form in simple solutions containing an excess of Ca^{++}, all phosphate polymers studied here possess about the same calcium-binding capacity per mol of P (Ca/P approx. 0.5). Consequently differences in complex-stability must be responsible for those distinct differences in biological activity seen in figure 4. Although frequently stated in the chemical literature (Schmidt, 1951) that calcium complexes of orthophosphate and of its cyclic derivatives possess low stabilities, relative to those of the linear polymers, only a few of the dissociation constants appear to have been measured validly.

From preliminary observations in this laboratory (Gosselin, Tidball and Coghlan, in progress), values of relative stability can be estimated. With the ion-exchange method of Schubert *et al.* (1950) and using Ca^{45} of high specific activity, measurements were made of the polymer concentrations which were required to remove a standard amount of radiocalcium from its combination with a cation resin ($K_d = \frac{1}{2} K'_d$). In table 3 the concentration required of each compound is listed in mols of P per liter and in values relative to that for HMP. These observations were made at 37°C, pH 7.4, and ionic strength 0.16, to mimic

the physiochemical environment of mammalian cells. Insofar as the stability of its calcium complex is relevant, a compound's ability to produce hypocalcemia should be inversely proportional to the values in table 3. In these studies, Na-

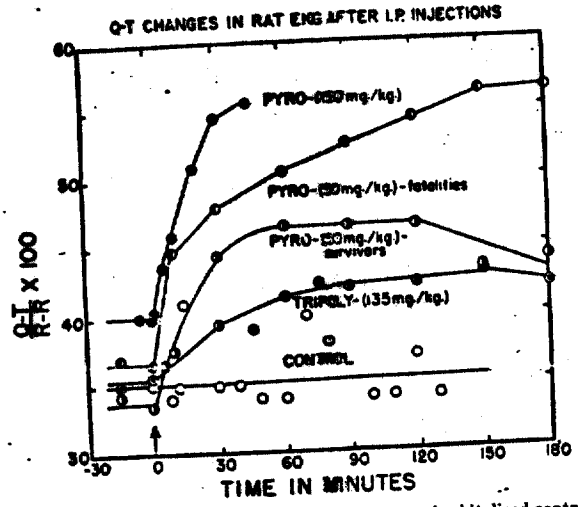

FIG. 5. Average changes in Q-T (relative to R-R) in a pentobarbitalized control rat and in rats which received at zero time a single intraperitoneal injection of sodium pyrophosphate or sodium tripolyphosphate. Doses are in mgm. P/kgm. Except for the control, each point is the mean of at least three rats.

TABLE 3

The relative dissociation of calcium-phosphorus complexes
(Temp. 37°C., pH 7.4, ionic strength 0.16)

COMPOUND	MOLAR CONC. P (WHEN Kd = 1Kd)*	MOLAR CONC. P RELATIVE TO HMP (=1)
Hexametaphosphate	1.3×10^{-4}	1.0
Tripolyphosphate	1.3×10^{-4}	1.0
Pyrophosphate	7.0×10^{-4}	5.3
Tetrametaphosphate	2.6×10^{-3}	20
Trimetaphosphate	9.5×10^{-3}	72
Orthophosphate	1.6×10^{-2}	120

* Method of Schubert, Russell and Myers (1950).

HMP and tripolyphosphate had the greatest potency per mol of P and orthophosphate the least; pyrophosphate was much stronger than the two cyclic polymers.

DISCUSSION. Prolongations of the Q-T interval in the ECG, prior to the de-

development of abnormalities in rate and rhythm, have been presented as evidence of reductions in the extracellular level of ionized calcium in rats receiving polymeric phosphates. Although hypopotassemia may produce a somewhat similar change in the ECG (Ernestene and Proudfit, 1949), potassium salts of these compounds, like their sodium salts, are recognized to be almost completely ionized in dilute solutions. When given to rats by the *intravenous* route, the potency of each material in producing hypocalcemia (figure 4) could be correlated in a gross way with the relative stability of its calcium complex *in vitro* (table 3). Deaths which occurred during i.v. infusions of Na-HMP, Na pyrophosphate, and Na tripolyphosphate are best ascribed to the cardiac consequences of this hypocalcemia. The weaker complexing (or chelating) agents, ortho-, trimeta-, and tetrametaphosphate, had no significant effect on the Q-T or heart rate, at least during the first ten minutes of these infusions. Severe hypocalcemia due to the intravenous administration of sodium orthophosphates has been reported, however, in dogs (Binger, 1917) and in cats (Koenig *et al.*, 1952), and so the negative results seen here may have been due to an insufficient dose.

When given by the *intraperitoneal* route, neither Na-HMP nor Na tripolyphosphate produced unequivocal ECG evidence of hypocalcemia during a two-hour period after LD_{50} doses. At least two factors may have obscured it: the mobilization of Ca^{++} rapidly from bone or other depots, and the simultaneous development of a metabolic acidosis. It is generally recognized (Binger, 1917; Shohl, 1939) that acidosis affords some protection against the physiological disturbances of hypocalcemia. The combination of acidosis and pentobarbital anesthesia may have been responsible for the absence of hypocalcemic tetany in these rats. Of the compounds tested intraperitoneally, only pyrophosphate produced significant hypocalcemic disturbances of the heart; figure 5 suggests that its relatively high intraperitoneal toxicity was a consequence of this action. Since the pyrophosphate complex was not unusually stable *in vitro* (table 3), perhaps high blood concentrations were responsible, i.e., this compound must have been mobilized from the peritoneal cavity more rapidly, relative to its rate of hydrolysis or excretion, than was HMP or tripolyphosphate. It also appears probable that the enzymatic hydrolysis of HMP and tripolyphosphate did not furnish appreciable amounts of circulating pyrophosphate.

In contrast to hypocalcemia, an acidosis followed the administration of HMP whatever the route and whatever the associated cation. This acidosis arose from the hydrolysis of the polymer into phosphoric acid residues. From the data in figure 1, the amount of acid released *in vivo* was readily estimated whenever the extent of hydrolysis was known from data on phosphate excretion (Gosselin *et al.*, 1952). After 29 mgm. P as i.p. Na-HMP, the urine of injected rats contained 0.31 meq. of fixed acid in excess of that by fasting controls (table 2). Since the theoretical value was 0.45 meq. H^+, a urinary loss of fixed base must have accompanied this acidosis; this conclusion is consistent with the observation that the plasma CO_2 content of treated rats failed to return to that of fasting control rats, even after two days. Because of the speed at which HMP was hydrolyzed *in vivo*, renal excretion was too slow to prevent a systemic acidosis. Observed reductions

in the CO_2 content of arterial plasma (figure 2) suggest that this acidosis was a major cause of death after an LD_{50} dose (192 mgm. P/kgm.). This dose is estimated to have produced 3.9 meq. H^+ per kgm., a value which is approximately the LD_{50} of intraperitoneal acetic acid and of hydrochloric acid in the rat (unpublished data by one of us, H. C. H.). Presumably an acidosis was also responsible for deaths after large doses of Ca-H_3P; its low toxicity may be attributed to a slow mobilization from sites of injection.

Sodium tripolyphosphate in rats is even more extensively hydrolyzed than Na-HMP (Gosselin *et al.*, 1952), and although its hydrolysis releases less acid (0.54 mol H^+ per mol P), equal doses of either compound are estimated to produce almost equal amounts of acid *in vivo*. In both cases deaths from intraperitoneal LD_{50} doses were presumably a consequence of this acidosis. A comparable acidosis from an LD_{50} dose of sodium pyrophosphate is highly improbable, however rapidly the compound may be split, because this lethal dose is so small and because so little acid is available from its hydrolysis at pH 7.4 (0.28 mol H^+/mol P). Because trimeta- and tetrametaphosphate are essentially unhydrolyzed in rats (Gosselin *et al.*, 1952), they can be administered parenterally, like sodium orthophosphate at pH 7.4, without significantly disturbing the acid-base balance. Their low toxicities are an expression of their inability to produce either acidosis or hypocalcemia under the conditions tested.

SUMMARY

Two severe biochemical disturbances have been identified in rats after the parenteral administration of a short-chain linear polymer of sodium phosphate (Na-HMP); they are (a) a reduction in the plasma concentration of ionized calcium and (b) a metabolic acidosis. Hypocalcemia was due to the formation *in vivo* of a chelate-complex between Ca^{++} and the HMP anion. Acidosis resulted from the enzymatic hydrolysis of the polymer into orthophosphoric acid residues. As identified by characteristic changes in the electrocardiogram, hypocalcemia could be detected when the sodium polymer (Na-HMP) was given by the intravenous route only, in which case cardiac arrest promptly occurred. A systemic metabolic acidosis resulted whatever the parenteral route of administration. As judged by reductions in the content of CO_2 in arterial plasma, this acidosis was regarded as a major cause of death after intraperitoneal doses of Na-HMP in the rat. Similar conclusions were reached with sodium tripolyphosphate. Sodium pyrophosphate owed its relatively high toxicity to its ability to evoke hypocalcemia, even by the intraperitoneal route. Least toxic were the stable compounds, ortho-, trimeta-, and tetrametaphosphate, which produced neither acidosis nor hypocalcemia. Using Ca^{45}, measurements were made of the relative stability of the calcium complexes formed *in vitro*.

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THE HYDROLYSIS AND EXCRETION OF POLYMERIC PHOSPHATE[1]

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Polymers of phosphoric acid have found extensive use in many detergents because they are able to form stable soluble complexes with most cations, thus serving as efficient water softeners. In addition to this industrial interest in polyphosphates, the biochemist's attention has been stimulated by the recent demonstration of polymeric metaphosphates in yeast, fungi and certain bacteria (Ingelman, 1950; Schmidt, 1951) but not in mammalian tissue. Although Kitasato (1928) demonstrated *in vitro* that tissues contain enzyme(s) capable of hydrolyzing polymeric metaphosphate, little attention has been paid to the metabolic fate of phosphate polymers when given to mammals.

The interest of this laboratory in polyphosphates arose from measurements of their ability to form highly stable complexes with the uranyl ion (Rothstein and Meier, 1951). On the expectation that these compounds might prove efficacious in the treatment of metal poisoning, preliminary observations (Gosselin *et al.*, 1951) were presented on the metabolic fate of phosphate polymers when injected in laboratory mammals; more extensive data are summarized here. These observations on rats and rabbits include the distribution, hydrolysis and excretion of sodium and calcium salts of four poly(meta)phosphates: trimetaphosphate, tetrametaphosphate, tripolyphosphate, and hexametaphosphate.

Materials and Methods: Samples of sodium trimeta-, tetrameta-, and tripolyphosphate (including the recrystallized hexahydrate of the latter) were generously furnished by the Victor Chemical Company. All these compounds are crystalline. On the basis of titration curves and conductivity data (Davies and Monk, 1949), trimeta- and tetrametaphosphate are generally regarded as cyclic polymers with phosphorus and oxygen in 6- and 8-membered rings, respectively. The existence of other cyclic phosphate polymers has not been proved (Davies and Monk, 1949). Tripolyphosphate (also called triphosphate) is known to be a linear or open-chain polymer (Monk, 1949).

In contrast sodium hexametaphosphate is a non-crystalline material (Partridge, 1949) believed to be a mixture of various molecular species, of which long linear phosphate chains are the principal component, if not the exclusive one (Van Wazer and Holst, 1950). Consequently the term hexametaphosphate is now regarded as a misnomer (Partridge, 1949). Since the name has been applied to many different phosphate glasses, it is an inadequate, if not misleading, designation for the material used in this study. The following observations by this laboratory represent a partial chemical characterization of commercial sodium hexametaphosphate (Einer and Amend); two other samples (including one from Victor Chemical Company) proved to be similar, though not identical.

The commercial sodium hexametaphosphate (Na-HMP) used here was a white powder

[1] This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, N. Y.

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freely soluble in water, containing 23.6 per cent P (by weight). For each mol of P, there were 1.22 mols of sodium and about 0.10 mols of some other cation, presumably dissociable hydrogen. By the use of an ion-exchange resin, the free hexametaphosphoric acid was prepared and titrated electrometrically with NaOH. An analysis of this titration curve by the method of Van Wazer (1950) indicated 6.0 atoms of P in an average molecule of the polymer mixture (an average weighted by its P content); this represents a mean molecular weight of 660. The material was not systematically fractionated. Presumably it contained appreciable amounts of tripolyphosphate (Bell, 1947a). Any cyclic polymers which may have been present were removed by the method of Davies and Monk (1949), but since the product so recovered did not differ consistently from the original material in terms of hydrolysis and excretion, no distinction between the two has been made in the text.

TABLE I

The incubation of heparinized rabbit blood with and without added phosphate polymer (see text for explanation)

	PLASMA CONC. MICROGR. P/ML.		
	ortho P	labile P	polymer P
No polymer added			
(a) original blood before incubation	38.0	1.3	0
(b) original blood after incubation	40.7	1.0	0
Sodium trimetaphosphate			
(c) trimeta. + blood before incubation	39.8	29.7	28.4
(d) trimeta. + blood after incubation	39.8	29.7	28.7
(e) theoretical	—	—	29.5
Sodium tetrametaphosphate			
(c) tetrameta. + blood before incubation	38.1	39.1	37.8
(d) tetrameta. + blood after incubation	38.3	39.7	38.7
(e) theoretical	—	—	38.8
Sodium tripolyphosphate			
(c) tripoly. + blood before incubation	35.2	—	—
(d) tripoly. + blood after incubation	43.5	7.3	6.3
(e) theoretical	—	—	6.4

Calcium salts of all four phosphate polymers were prepared by adding 0.5 mol $CaCl_2$ to solutions of the sodium salt containing one mol of P. By raising the pH above 4 (NaOH) and by adding ethyl alcohol (final conc. 25 per cent), over 90 per cent of the labile P was precipitated as a calcium salt. By analysis these precipitates contained Ca and P in a mol ratio of about 0.5. The fresh precipitates were resuspended in water and the pH of the suspensions was adjusted (with NaOH) to 7.4. Traces of alcohol (perhaps 2 per cent in the final product) served as a deflocculating agent. Washed precipitates of calcium hexametaphosphate (Ca-HMP) were also dried *in vacuo*, but such treatment was found to reduce markedly their solubility *in vitro* and to render them almost inert *in vivo*. In addition to these suspensions, a mixed calcium-sodium salt of tripolyphosphate was prepared by adding 0.2 mols of $CaCl_2$ to each mol of P; at a pH of 7.4 this material remained in solution and was employed as such. A comparable preparation of hexametaphosphate (Ca-Na-HMP) contained 0.3 mols of calcium per mol of phosphorus. All solutions and suspensions were adjusted with NaOH to a pH of 7.4 (glass electrode) and were injected within an hour of preparation.

In aqueous solutions and suspensions of these materials, one per cent or less of the phosphorus was present as orthophosphate. Although stable at room temperatures, these compounds in dilute solutions were completely hydrolyzed to orthophosphate within 7

minutes in the presence of 1.0 N HCl at 100°C.; this acid-hydrolysable fraction is called labile P. Analyses for orthophosphate were generally accomplished by a modification of the method of Fiske and Subbarow, in which alcohol was added to speed color development (Meyerhof and Oesper, 1947). In addition a method of Berke and Rothstein (unpublished data) has facilitated the analysis of small quantities of labile P in the presence of large amounts of orthophosphate. All dosages and concentrations are expressed in terms of phosphorus present as labile phosphate. Values of metaphosphate in serum and urine were based on the 7-minute hydrolysis of protein-free filtrates (trichloracetic acid) with appropriate corrections for pre-injection levels. It was shown that all polyphosphate remained in the filtrates after precipitation of plasma proteins by trichloracetic acid.

Prior to a study of blood levels *in vivo*, sodium salts of trimeta-, tetrameta-, and tripolyphosphate were added *in vitro* to heparinized rabbit blood. The distribution of these substances between cells and plasma could not be adequately examined by a direct analysis of washed red blood cells, because the latter proved rich in labile phosphate compounds even before exposure to polyphosphate. In contrast serum and plasma of fasting rabbits contained little labile P. Table 1 summarizes plasma concentrations of ortho- and labile phosphate (a) before and (c) 10 minutes after the addition of known amounts of polyphosphate to whole blood; included in these data are small corrections for dilution by the isotonic saline solution in which the polymer salts were initially dissolved. From the measured hematocrit (0.41) theoretical values (e) were calculated upon the assumption that the added polymeric P did not penetrate blood cells. Within analytical uncertainty (2 to 3 per cent in these duplicate phosphate analyses), the observed plasma concentrations were in agreement with these theoretical values (*cf.* c and e). Aliquots of the same blood-polymer mixtures were then incubated at 38°C. for 150 minutes; pH values of these incubated samples lay between 7.2 and 7.4. As seen in table 1, this incubation did not lead to a significant change in the plasma concentration of labile P in any blood sample containing polyphosphate (*cf.* c and d). This constancy rules out significant hydrolysis of the polymers during the incubation. As a result the distinct rises in plasma orthophosphate must be explained by leakage from the blood cells. The data suggest that blood cells exposed to polyphosphate leak orthophosphate more rapidly than control cells (*cf.* a and b with c and d). It is apparent that all three polyphosphate compounds behaved alike in these *in vitro* studies; in a similar test with hexametaphosphate, the same conclusions were reached. These polymers also proved stable in voided rat and rabbit urine, even when heavily contaminated with coliform bacteria.

Only adult male animals were used. Albino rats varied in body weight from 270 to 310 grams. Two rats occupied each all-glass metabolism cage. Rabbits were fasted from 18 to 24 hours before use; rats were deprived of feed only after dosage with polyphosphate, and occasionally they were allowed a standard chow ration during the experiment. In most cases uninjected controls were observed simultaneously.

Results: In metabolic studies the two cyclic polymers proved to be similar to one another but distinctly different from the two linear polymers. Observations on cyclic compounds will be presented first.

Trimeta- and tetrametaphosphate: Figure 1 demonstrates serum concentrations of ortho- and labile phosphate in two adult rabbits after the intravenous injection of sodium trimetaphosphate (curves A) and of sodium tetrametaphosphate (curves B). In fasting rabbits serum phosphate levels remained essentially constant during pre-injection periods of more than an hour. Control values of labile P have been subtracted from each datum in the figure. Both compounds were rapidly removed from the plasma at rates which were approximately exponential. By back-extrapolation of these curves to the time of injection, the short-term volume of distribution of trimetaphosphate was calculated to be 12

per cent of the body weight; that of tetrametaphosphate appeared to be somewhat greater. Neither compound produced changes in the serum concentration of orthophosphate until late in the experiment, when small but significant upward trends were noted.

Fasting rabbits were anesthetized with pentobarbital and catheterized for complete urine collections. During water diuresis, the excretion of orthophosphate remained relatively constant in any one rabbit but varied between 30 and 100 microgm. P/min. among a group of animals; labile P was generally not detectable during these control periods. Figure 2 represents the cumulative uri-

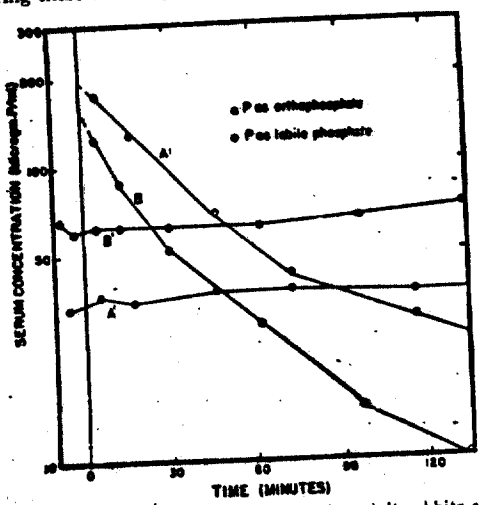

FIG. 1: Serum levels of ortho- and labile P in fasting adult rabbits anesthetized with pentobarbital. At zero time, rabbit A received i.v. sodium trimetaphosphate (23.2 mgm. P/kgm.); rabbit B received i.v. sodium tetrametaphosphate (24.2 mgm. P/kgm.). Pre-injection values of labile P have been subtracted from each datum in the top curves (7 and 0.4 in A and B, respectively).

nary excretion of labile P (expressed in per cent of the dose) after the intravenous administration of sodium trimetaphosphate (curve A) and of sodium tetrametaphosphate (curve B). Both compounds appeared promptly in the urine, but after three hours only small amounts were detectable, in conformity with plasma concentrations. Consistent with its more rapid plasma clearance, tetrametaphosphate was here excreted more rapidly than trimetaphosphate. The excretion of orthophosphate also rose following these injections. For example, rabbit A excreted about 23 microgm. ortho-P/min. during a pre-injection period of an hour; following intravenous trimetaphosphate, the rate rose to 83, 56, and 44 microgm. ortho-P/min. in three consecutive 30-minute periods. With rabbit B, the rise was less marked and probably not significant. In both instances rates

of orthophosphate excretion returned to pre-injection levels within two hours. Although trimetaphosphate produced a significant rise in the rate of orthophosphate elimination, this increment was small relative to that of labile phosphate; no more than 2 per cent of the administered polymer could be accounted for as extra orthophosphate in the urine.

Urinary excretion was also observed in fasting rats which received by various routes sodium and calcium salts of trimeta-, tetrameta-, and tripolyphosphate. As tabulated in table 2, each datum represents that percentage of the P ad-

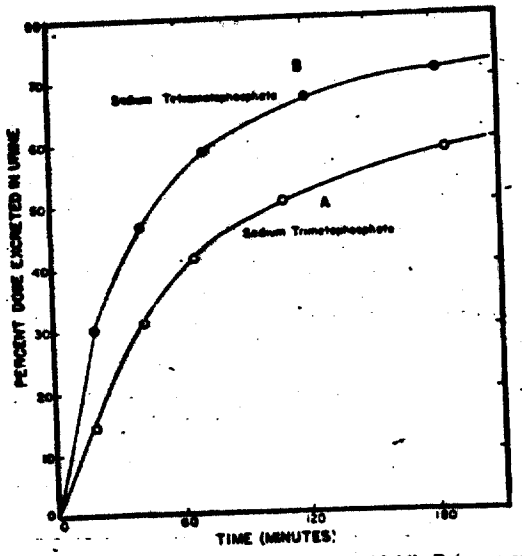

FIG. 2: Curve A is the cumulative urinary excretion of labile P (expressed in per cent of the dose) by a fasting rabbit, which received intravenously at zero time 25.2 mgm. P/kgm. as sodium trimetaphosphate. Curve B similarly resulted from 24.2 mgm. P/kgm. as sodium tetrametaphosphate. No labile P was detectable in pre-injection samples of urine.

ministered as polymer which was recovered within 24 hours in the urine as labile P, ortho-P, or both. Since uninjected control rats excreted almost no labile P (less than 0.01 mgm.), the labile P observed in the urine of treated rats is presumed to have been unhydrolyzed polymer. Except when given by the oral route, trimeta- and tetrametaphosphate promoted a high excretion of labile phosphate, amounting to between 48 and 90 per cent of the dose in the first 24 hours and an additional 2 to 5 per cent in the second 24 hours. Urinary recoveries of labile phosphate were consistently higher when these polymers were given as sodium rather than as calcium salts. As judged by the first 24 hours, the calcium salt of tetrametaphosphate appeared to be excreted more rapidly when given

intraperitoneally than subcutaneously, but this distinction was not apparent in the excretion of trimetaphosphate. By the oral route no more than 4 per cent of either compound was recovered as urinary labile phosphate.

To estimate the amount of orthophosphate derived from these doses of polymer, it was necessary to define the orthophosphate excretion of control rats. Probably because rats were not deprived of food until they entered metabolism cages, excretion in the first day was rather variable within the range of 10 to 20 mgm. P; the mean of 24 control rats was 14.9 (± 0.76) mgm. P. In column B

TABLE 2

*Urinary excretion of polymer phosphorus in the rat**

COMPOUND	SALT	ROUTE	NO. RATS	MEAN 24-HOUR EXCRETION IN PER CENT OF DOSE		
				A labile P	B† excess ortho-P	A + B total P
Trimetaphosphate	Na	i.p.	4	89	0	89
	Ca	i.p.	2	56	10‡	66
	Ca	s.c.	4	62	0	62
	Na	oral	2	2	38	40
Tetrametaphosphate	Na	i.p.	4	90	12‡	102
	Ca	i.p.	4	77	3‡	80
	Ca	s.c.	4	48	0	48
	Na	oral	2	4	18	22
Tripolyphosphate	Na	i.p.	6	9	66	75
	Ca-Na	i.p.	6	8	76	84
	Ca	i.p.	4	0.5	39	40
	Ca	s.c.	6	0.6	25	26

* All rats were males of about 300 gm., given water *ad libitum* but not food. The polyphosphates were injected once in doses which varied between 15.5 and 33.5 mgm. P per rat. Data represent mean cumulative excretions during the 24 hours following administration of these compounds.

† Column B records average ortho-P excretion in excess of average control, the difference expressed in per cent of dose. Control excretion by 24 uninjected rats averaged 14.9 mgm. ortho-P (standard error 0.76) and essentially no labile P (.01 mgm.).

‡ As explained in the text, these values are not significantly different from zero.

of table 2, the difference between the urinary orthophosphate of treated rats and that of the average control is expressed in per cent of the dose. Because the experimental groups were small, large errors were inevitable in this method of estimating the percentage of polymer P excreted as orthophosphate. The data serve to demonstrate, however, that no significant amount of orthophosphate was derived from these parenteral doses of trimeta- or tetrametaphosphate, since the orthophosphate excretion in these 22 rats was in every instance within the range of controls and averaged the same as controls (14.5 and 14.9 mgm. P, respectively). When these two compounds were administered by stomach tube, however, the excretion data in table 2 indicate that appreciable amounts of polymer were hydrolyzed to orthophosphate. Even after intraperitoneal ad-

ministration, a small but significant hydrolysis could be demonstraed by employing doses much greater than those in table 2. For example, after an i.p. dose of 167 mgm. P as sodium trimetaphosphate, 81 per cent of the phosphorus was recovered as labile P in the urine within 24 hours; another 6 per cent appeared as extra orthophosphate, represented by 10 mgm. P in excess of that found in any control sample. Although less convincing with large doses of tetrametaphosphate, an elevation in orthophosphate excretion was also apparent. Because these data cannot be explained by orthophosphate contamination of the original polymer solutions, they constitute presumptive evidence that rats, like rabbits, hydrolyze a small fraction of injected trimetaphosphate (and perhaps of tetrametaphosphate).

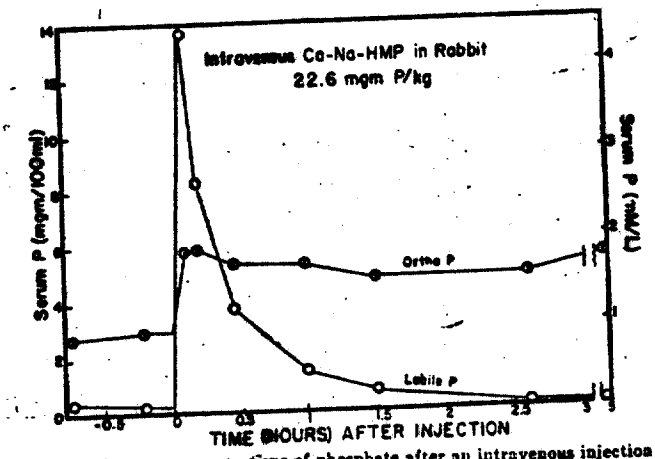

FIG. 3: Blood serum concentrations of phosphate after an intravenous injection of Ca-Na-HMP (Ca/P = 0.30), given to fasting rabbit anesthetized with pentobarbital.

Hexametaphosphate and tripolyphosphate: By the oral route, these two linear polymers proved similar to the ring compounds. When sodium or calcium salts were given by stomach tube to rats and rabbits, only trace amounts of labile P appeared in the urine; the urinary excretion of extra orthophosphate was significant but represented only a small fraction of the dose. After parenteral administration, however, a prominent metabolic distinction between the linear and cyclic polymers became apparent.

After a single intravenous injection of hexametaphosphate (Ca-Na-HMP) in a fasting rabbit (figure 3), the serum labile P level rose immediately and then decreased rapidly, reaching control levels in about two hours. Serum orthophosphate concentration also showed a prompt rise and often remained high for 24 hours, presumably the result of *in vivo* hydrolysis of the polymer. Five rabbits received this preparation intravenously in doses of 20 to 30 mgm. P/kgm. In

all cases the serum concentration of labile P fell at an exponential rate with a "half-time" of 10 to 15 minutes, but thereafter the plasma clearance became gradually slower. By back-extrapolation of the serum concentration to the time of injection, the volume of distribution of Ca-Na-HMP was variously estimated between 10 and 13 per cent of the body weight (five rabbits). When HMP was given intraperitoneally, the rises in plasma concentration were slower, less pronounced, and more prolonged (figure 4). Blood levels were not observed after the administration of tripolyphosphate.

In contrast to the cyclic polymers, parenteral doses of both hexametaphosphate and tripolyphosphate produced prompt and sustained rises in orthophosphate

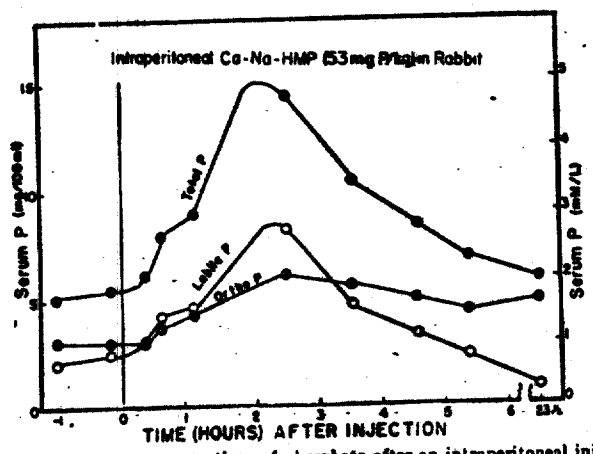

FIG. 4: Blood serum concentrations of phosphate after an intraperitoneal injection of Ca-Na-HMP, given to a fasting anesthetized rabbit.

excretion by fasting rabbits (figure 5). Relatively small amounts of labile P also appeared in the urine. Within 2.5 hours, almost 70 per cent of an intravenous dose of tripolyphosphate was recovered as urinary phosphate, but less than a third of this amount was in the form of labile P (figure 5). Similarly after HMP, labile P excretion ceased within a few hours, in accordance with plasma levels, but the excretion of extra orthophosphate continued for 24 hours or more. About 30 per cent of HMP given parenterally (20–30 mgm. P/kgm.) was accounted for as labile P in the urine, the rest eventually appearing as orthophosphate (four rabbits). Attempts to estimate the plasma renal clearance of HMP in rabbits proved unsatisfactory, perhaps because of different clearance values for different size-fractions of the polymer population.

In rats, parenteral doses of these linear polymers also promoted a large excretion of orthophosphate and correspondingly less labile P than did the cyclic compounds. Twenty rats out of 22 receiving tripolyphosphate (table 2) and 60

rats out of 66 receiving HMP (table 3) excreted more orthophosphate than the highest control value (19.8 mgm. P). Table 2 offers a comparison between tripolyphosphate and the ring polymers in terms of daily phosphate excretion in rats. With HMP a more extensive study of excretion was undertaken to define the influence of dosage, route of administration, and calcium content. Average values are summarized in table 3, together with expressions of vari-

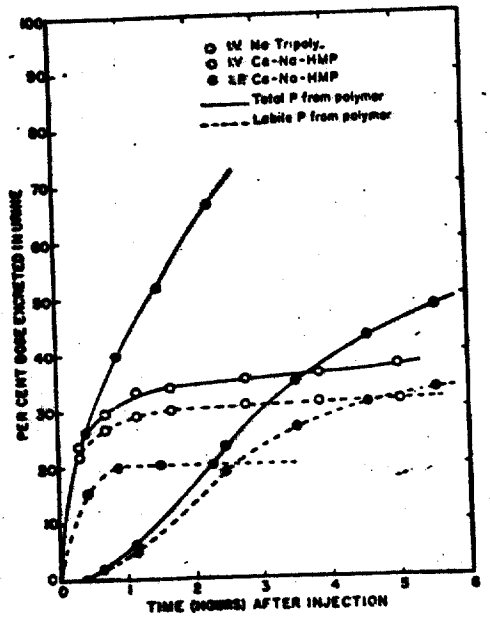

FIG. 5: The cumulative urinary excretion by fasting adult rabbits of phosphorus derived from injected linear polymers. The total P represents the cumulative excretion of both labile P and ortho-P, in excess of the mean excretory rate during a pre-injection period of more than an hour. The doses were as follows: intravenous sodium tripolyphosphate 12.5 mgm. P/kgm.; i.v. Ca-Na-HMP 22.6 mgm. P/kgm.; i.p. Ca-Na-HMP 33 mgm. P/kgm.

ance. Although several of the groups were small, the means are judged adequate to define the general pattern of excretion.

On the premise that labile P in the urine of injected rats was unhydrolyzed HMP, table 3 demonstrates that 15 to 20 per cent of intraperitoneal Na-HMP was recovered in the urine within 24 hours (viz. B, C); even less, percentagewise and absolutely, was observed after doses much in excess of 110 mgm. P/kgm. (viz. D). The excretion of intact polymer was also less when calcium preparations of HMP were employed instead of the sodium salt; thus mean values were 4 per cent of Ca-HMP (F) and 11 per cent of Ca-Na-HMP (mean of 13 pairs of

female rats). This contrast between intraperitoneal Na-HMP and Ca-HMP proved to be related to the route of injection; the distinction was essentially reversed after subcutaneous administration. For example, urinary labile P from s.c. Ca-HMP (G, H, J) was three to four times greater than from i.p. Ca-HMP; the former averaged 15 to 17 per cent of the dose, even at doses as high as 160 mgm. P/kgm. (J). Subcutaneous Na-HMP, however, was poorly tolerated by rats and only small amounts of labile P were recovered in the urine (E). Estimates of the total amount of P derived from HMP were furnished by the differences in urinary P (ortho plus labile) between treated groups and fasting controls. As seen in table 3, about 80 per cent of the P given as Na-HMP was represented in the first day's urine (B, C, E), but only 35 to 45 per cent of the

TABLE 3

Daily urinary excretion of ortho and labile phosphate by fasting rats given HMP*

group	no. rats	salt of HMP	dose mgm. P	route	excretion in mgm. P per day 24-hour: ortho P ± S.D.	excretion in mgm. P per day 24-hour: labile P (range)	excretion in per cent of dose: 24-hour: labile	24-hour: excess ortho	24-hour: total†	48-hour: labile	48-hour: excess ortho	48-hour: total†
A	12	none			14.9 ± 3.3	0.0 (0)						
B	7	Na	15.6	i.p.	25.0 ± 3.8	3.0 (2.0–3.8)	19	65	84			
C	8	Na	29.5	i.p.	32.9 ± 2.7	5.1 (2.7–8.0)	17	61	78	17	79	96
D	3	Na	45.0	i.p.	34.2	1.5 (1.0–1.8)	3	43	46			
E	2	Na	31.6	s.c.	39.5	1.3 (1.2–1.4)	4	78	82	4	88	92
F	4	Ca	31.6	i.p.	25.4 ± 3.3	1.3 (0.7–1.9)	4	33	37	5	50	55
G	2	Ca	15.8	s.c.	19.4	2.3 (2.2–2.3)	15	28	43			
H	6	Ca	31.6	s.c.	21.7 ± 2.1	5.0 (4.5–5.8)	16	21	37	17	35	52
J	1	Ca	47.4	s.c.	32.3	8.0	17	37	54			

* All rats were males with body weights lying between 270 and 310 grams. They were injected once at the beginning of the fast. Water was drunk *ad libitum*.

† Represents the sum of ortho- and labile P, minus that in the control group (A), expressed as a per cent of the P given as HMP.

P from Ca-HMP was excreted in this interval (F, G, H). Both the intraperitoneal and subcutaneous routes were identical in this regard, in spite of their contrasting influences on the excretion of labile P. Urines collected during the second 24-hour period contained only small amounts of labile P, averaging 0.3 to 0.4 mgm. after Ca-HMP and less than 0.1 mgm. after Na-HMP; beyond 48 hours, values were below the limits of reliable analysis. Orthophosphate excretion also continued above control levels in the second 24 hours, the difference totaling 4 to 5 mgm. (C, E, F, H). In consequence over 90 per cent of the P from Na-HMP appeared in the urine within 48 hours (C, E), but scarcely 50 per cent of Ca-HMP was recovered in this interval (F, H). Many of these data were corroborated by studies with rats feeding on a standard chow ration.

The subcutaneous site of Ca-HMP behaved as a true depot, a nodule palpable for many weeks, eventually becoming a collection of dense connective tissue in which mineral salts were embedded. For over a week these nodules contained

several times more labile P than ortho-P, as judged by acid extractions of minced nodules. Because these extractions were found to be incomplete, phosphate released by wet-ashing was adopted as a better measure of residual HMP in the dissected nodule. By this criterion, HMP in the nodule decreased by 30 per cent in 24 hours, 51 per cent in 5 days, and 83 per cent in 11 days, decrements which were somewhat smaller than anticipated from the more reliable data on excretion. These observations demonstrate that most of the delay in the urinary excretion of Ca-HMP was due to its slow mobilization from the site of injection. Although considerable amounts of polymeric P persisted at the site of injection for over a week, mobilization was so slow after the first 48 hours that no detectable amounts could escape into the urine before hydrolysis.

Attempts to demonstrate fecal excretion of HMP proved unsuccessful. In rats feeding *ad libitum*, parenteral doses of HMP consistently produced a marked fall in total fecal P (ashed samples), presumably because of a reduction in food intake. With fasting rats, neither before nor after the parenteral administration of HMP was labile P detected in acid extracts of fresh feces. Although experimental samples sometimes contained more acid-extractable orthophosphate than control samples, the difference was never large. From these data it seems improbable that appreciable amounts of HMP were excreted by the intestinal route.

Discussion: Enzymes were presumably responsible for all hydrolyses observed after parenteral doses of these phosphate polymers, since in neutral solutions *in vitro* all four compounds were virtually stable for more than 24 hours at 37°C. Although the extensive enzymatic degradation of the linear polymers served to distinguish them from the cyclic compounds, the latter were not completely stable *in vivo*. Thus parenteral doses of trimetaphosphate stimulated the excretion of orthophosphate in amounts equivalent to a few per cent of the dose. These data constitute presumptive evidence for trimetaphosphatase activity in both rats and rabbits; the evidence for tetrametaphosphatase was less satisfactory. With this possible exception, no prominent difference was observed in the metabolism of the two cyclic polymers. In both cases phosphatase activity was so low, relative to the rate of polymer excretion, that these compounds were recovered largely intact in the urine. Significant quantitative differences, however, were apparent between the two linear polymers. Although both were excreted principally as orthophosphate, considerably more urinary labile P was recovered after salts of hexametaphosphate than after those of tripolyphosphate.

The enzymatic degradation of polymeric phosphate is generally assumed to be an end-hydrolysis, in which case the extent of hydrolysis may be gauged by the production of orthophosphate. Ingelman and Malmgren (1947) have demonstrated, however, that certain fungi attack random P-O-P linkages in high-weight metaphosphate, not exclusively end-groups. If internal polymer hydrolysis occurs in mammalian tissue, its detection must await specific methods of microanalysis. With present methods, there is no assurance that the labile P observed *in vivo* was identical with the original polymer. For example, if the enzymatic depolymerization of HMP yielded trimetaphosphate, as Bell (1947b) believes to occur during the thermal hydrolysis of HMP, this cyclic derivative may have

been one of the prominent excretory products of HMP. There is much less difficulty in interpreting metabolic observations on the cyclic polymers. Although alkali slowly opens the trimetaphosphate ring *in vitro* to produce tripolyphosphate (Bell, 1947b), the excretion data in table 2 effectively deny this conversion *in vivo*.

Tissue assays for polyphosphatase activity were not specifically investigated in this study. Blood and urine were found to possess no hydrolytic activity against these polymers, but enzyme hydrolysis in the gastrointestinal tract was suspected by the failure to recover appreciable amounts of labile P in the urine after oral doses. Barnabee *et al.* (1950) have observed the hydrolysis of hexametaphosphate when incubated with powdered rat intestine. Kitasato (1928) has demonstrated the hydrolysis of "hexametaphosphate" *in vitro* by ground rabbit liver and kidney but not by striated muscle. In contrast Ingelman and Malmgren (1949) have promised data showing that their rabbit liver extracts possessed no hydrolytic activity against a linear polymer with a high molecular weight, although trimetaphosphate was readily converted to orthophosphate. Mattenheimer (1951) observed that extracts from several animal tissues hydrolyzed open-chain polymers but that none hydrolyzed the cyclic tetrametaphosphate, although trimetaphosphate was slowly split by extracts of rat liver and human kidney. At the moment it is impossible to reconcile these observations with each other or with current studies on intact rats and rabbits.

SUMMARY

Two linear phosphate polymers were extensively hydrolyzed to orthophosphate in rats and rabbits. In contrast two cyclic polymers suffered almost no hydrolysis and were promptly recovered as intact molecules in the urine.

Studies on linear or open-chain compounds showed that tripolyphosphate was more rapidly and more extensively degraded *in vivo* than its homologue with six atoms of phosphorus (labelled hexametaphosphate). The dose, route of administration, and associated cation all influenced the extent to which unhydrolyzed polymer escaped in the urine. Sodium salts of tripolyphosphate and hexametaphosphate were more rapidly mobilized from sites of injection and provided larger amounts of polymeric phosphate in the urine than did the corresponding calcium salts.

Although the cyclic polymers trimeta- and tetrametaphosphate were excreted virtually unchanged after parenteral administration, a small fraction of injected trimetaphosphate was converted to orthophosphate in rats and rabbits. All four polymers appeared to be hydrolyzed when given by the oral route.

Tissue sites of these enzymatic hydrolyses were not identified; no activity was present in blood or urine.

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Arzneim.-Forsch. 8: 286-89 (1958) 355

Zusammenfassung:

An 8 Ödemkranken und 3 Normalpersonen wurde die Wirkung von n-Chlorphenylbiguanidin auf die Ausgabe von Phosphaten, Bicarbonaten und Harnstoff untersucht. Die Wasserstoffionenkonzentration des Harns und des Plasmas und der Bicarbonatgehalt des Plasmas wurden gemessen. Die Ausscheidung von Bicarbonaten wurde vermindert. Eine deutliche Veränderung der Ausscheidung der übrigen untersuchten Substanzen wurde nicht festgestellt.

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Summary:

Urinary excretion of phosphates, bicarbonate and urea was investigated in 8 patients suffering from edema and in 3 normal controls. The plasma and urine hydrogen ion concentration and the plasma bicarbonate content were also measured. Excretion of bicarbonate was found to be reduced. No marked change was found in the excretion of the other substances tested.

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aus der Universitätsklinik in Düsseldorf:

Sind Orthophosphat und Polyphosphate bei chronischer Verfütterung von unterschiedlicher Verträglichkeit?

Von Professor Dr. F. Hahn, Dr. H. Jacobi und Dr. E. Seifen

Im Hinblick auf die Frage, ob die Verwendung von kondensierten Phosphaten und im besonderen von Polyphosphaten als Nahrungsmittelzusätze gesundheitlich als unbedenklich anzusehen ist, ist in allererster Linie zu klären, ob die Polyphosphate ernährungsphysiologisch anders zu werten sind als Orthophosphat, das einen normalen und notwendigen Bestandteil unseres Körpers und unserer Nahrung darstellt. Denn die Möglichkeit, daß die verschiedenen Phosphate sich in ihrer resorptiven Wirkung qualitativ nicht voneinander unterscheiden, ist durchaus gegeben, da die Polyphosphate im Darm gespalten und wahrscheinlich nur in gespaltener Form, d. h. als Orthophosphat, resorbiert werden (Jones [5]; Schreiern, Nöller [8]; Van Esch, Vink, Wit u. Van Genderen [3]). Im Falle einer vollständigen Spaltung wäre auch eine quantitativ gleiche Wirkung möglich. Allerdings spielt nach Lang u. Mitarb. [6] auch der Ort der Spaltung eine Rolle, da im Dickdarm durch Bakterien gebildetes Orthophosphat zum größten Teil nicht mehr resorbiert werde. Hinsichtlich der Vollständigkeit der Spaltung im Darm gehen die Anschauungen noch auseinander. Nach Lang u. Mitarb. [6], die eine unvollständige Spaltung annehmen, erscheint die Resorption der hochmolekularen Polyphosphate Grahamsalz und Kurrolsalz (Kaliumpolyphosphat) gering und auch Schreiern, Nöller [8] finden eine geringere Phosphataufnahme nach Grahamsalz als nach Pyro- und Tripolyphosphat. Andererseits deckt sich in den Versuchen von Van Esch, Vink, Wit u. Van Genderen [3] die von ihnen chemisch nachgewiesene weitgehende Spaltung von Polyphosphaten mit dem Befund einer qualitativ und quantitativ übereinstimmenden toxikologischen Wirkung, abgesehen vielleicht von einer etwas größeren Nierenschädigung nach sehr hohen Orthophosphat-Dosen. Dieser letzte Befund ist von Interesse, weil er die Möglichkeit andeutet, daß bei excessiv hoher Phosphat-Belastung über lange Zeit die Polyphosphate sogar weniger nachteilige Folgen haben könnten als Orthophosphat. Diese Tatsache überrascht vielleicht, weil man gewohnt ist, körperfremde Stoffe als bedenklicher anzusehen als körpereigene. Aber sie ist leicht verständlich, wenn man nach Polyphosphatzufuhr eine etwas verzögerte Phosphatresorption infolge der Notwendigkeit einer vorausgehenden Spaltung annimmt. Im übrigen sind Polyphosphate nicht organismenfremd, da sie in niederen Lebewesen vorkommen (Lit.: Lohmann u. Langen [7]). Das niederste Phosphat dieser Reihe, nämlich das Pyrophosphat, findet sich auch im Säugetierorganismus.

Die aufschlußreichen Versuche Van Esch, Vink, Wit u. Van Genderen [3] wurden mit einem Mischphosphat, bestehend aus ⅔ Pyrophosphat und ⅓ Kalium-Kurrolsalz durchgeführt. Um die Wirkung verschiedener Kondensationsstufen der Mono-Phosphorsäure getrennt kennen zu lernen, wurden von uns chronische Fütterungsversuche mit Ortho-(= Mono)-phosphat, Pyro-(= Di)-phosphat, Tripoly-(= Tri)-phosphat und dem bisher auch in der Fachliteratur fälschlich als „Hexametaphosphat" bezeichneten Polyphosphat Grahamsalz vorgenommen.

Methodik

Die Versuche wurden an gewöhnlichen Zuchtratten durchgeführt, die in Gruppen von meist 34–36 Tieren – in einigen Fällen auch weniger – mit Sherman-Diät, der die Phosphate in Konzentrationen von 1,8 % und 5 %, bezogen auf das Frischgewicht, zugesetzt waren, gefüttert wurden. Die Kontrollen erhielten die gleiche Kost ohne Phosphatzusatz. Für die 5- und 2%ige Phosphatkonzentration verwendeten wir sowohl Gruppen von männlichen als auch Gruppen von weiblichen Tieren, für die 1,8%ige Konzentration nur männliche Tiere. Die Fütterungsdauer betrug 6 Monate. Das Anfangsgewicht der Tiere war 50–60 g, während bis 70 g. Die Futteraufnahme wurde täglich, das Gewicht alle 7 Tage geprüft. Am Ende der Versuchsperiode wurde der Blutbild zweimal im Abstand von 8 Tagen bestimmt. Ein Teil der Tiere wurde seziert, die Organe gewogen und zur histologischen Untersuchung eingelegt*). Paraffinschnitte von den Organen wurden mit Hämalaun-Eosin gefärbt. Ionisierbares bzw. ionisiertes Calcium wurde mit Schwefelsäure nachgewiesen, wobei Kalkvorkommen als Calciumsulfatnadeln sichtbar werden. Der Eisennachweis wurde mit der Turnbullblau-Methode geführt. Bei einem weiteren Teil der Tiere nahmen wir nach Gesamtveraschung eine Calcium-, Phosphat-, Eisen- und Kupferbestimmung vor.

Die verwendeten Salze waren Dinatriumphosphat ($[4NaPO_3]_4 \cdot H_2O$; pH 6,1; 69,6% P_2O_5; 30,4% Na_2O), Natriumpyrophosphat ($Na_4P_2O_7$; pH 10,2; 53,3% P_2O_5; 46,7% Na_2O), Natriumtripolyphosphat ($Na_5P_3O_{10}$; pH 9,8; 57,8% P_2O_5; 42,2% Na_2O) und Grahamsalz ($[NaPO_3]_n \cdot H_2O$); pH 6,1; 69,6% P_2O_5; 30,4% Na_2O).

Die Futterplätzchen wurden aus einem Gemisch von 200 g Weizenschrot, 300 g Vollmilchpulver und 40 ml Wasser hergestellt und 8 Std. bei 60° C getrocknet. Die Plätzchen enthielten analytisch 1,43% P_2O_5, 0,43% CaO und 0,199% MgO.

Zur chemischen Analyse wurde eine Kombination der in Hoppe-Seyler's Handbuch [4] beschriebenen nassen Aufschlußverfahren vorgenommen. Zu diesem Zweck wird die getrocknete und völlig zerkleinerte Rasse in ein großes Becherglas, das 400 cm³ konz. HCl und 100 cm³ dest. Wasser enthält, gegeben. In der so verdünnten Säure werden die Tiere bis zur völligen Auflösung gekocht, ein vorheriges Zerkleinern der Tiere hat sich nicht bewährt.

Nach dem Abkühlen der Lösung wird unter ganz schwachem Erwärmen in kleinen Portionen (Spatelspitze) 10 g $KClO_3$ zugegeben. Danach läßt man schwachem Sieden halten, ausschließend erneut abkühlen. In die fast kalte Lösung wird in Anteilen erst 30 cm³ Perhydrol und schließlich 30 cm³ conc. HNO_3 gegeben, wobei wieder sehr langsam erwärmt und nach jeder Zugabe stark gekocht wird. Nun wird die Lösung erneut in schwachem Sieden erhalten, bis sie hellgrünlichblau bleibt und sich das Fett zusammenballt. Nach Beendigung des Aufschlusses soll die Lösung etwa 350 cm³ haben. Am nächsten Tag wird die vollkommen erkaltete Lösung in einen 500 cm³ Meßkolben filtriert, das Fett in einem Tiegel mit dem verwendeten Filtrierpapier trocken verascht und die Asche in verdünnter Salzsäure (1 : 1) gelöst und damit der Meßkolben aufgefüllt. Eisen-, Calcium- und Phosphatbestimmung nach Bitz [1], Kupferbestimmung nach Bode [2].

Ergebnisse:

Da nicht alle Versuche auf einmal durchzuführen waren, gliedern sich die gesamte Versuchsdauer von etwa 2 Jahren in mehrere Versuchsabschnitte. Jeder Abschnitt (im folgenden mit den Buchstaben A bis H gekennzeichnet) bestand aus einer Kontrollgruppe für jedes Ge-

*) Für die Durchführung der histologischen Untersuchungen sagen wir Herrn Prof. Dr. Dr. A. Kriesselbach, Direktor des Pathologisch-anatomischen Instituts der Medizinischen Akademie Düsseldorf, und Herrn Dr. C. Lindner auch an dieser Stelle unseren Dank.

Phos

schlecht und einigen Phosphatgruppen, die entweder nach der Art der Phosphate oder nach der Konzentration variiert waren. Die Zahl der Gruppen für die einzelnen Versuchsabschnitte war verschieden. In dem Abschnitt D, bestehend aus einer Kontrollgruppe und nur einer Gruppe weiblicher Tiere mit 5% Grahamsalz brach am Versuchsbeginn eine Seuche aus, welche die 36 Kontrollen auf 14, die 36 Phosphattiere auf 26 reduzierte und eine Verschiebung der Mittelgewichte beider Gruppen zugunsten der Kontrollgruppe veranlaßte, wodurch eine Auswertung der Wachstumskurven unmöglich wurde. Die übrigen Ergebnisse, die mit diesem Versuch erhalten wurden, sind jedoch berücksichtigt.

Die Auswertung der Wachstumsversuche erfolgte in Abb. 1 in der Form, daß die mittleren Gewichtsdifferenzen zwischen den Kontrollen und den mit Phosphat gefütterten Tieren alle 3 Wochen errechnet und in Prozent des Kontrollgewichtes angegeben wurden. Man erkennt, daß ein 5%iger Phosphatzusatz eine deutlich stärkere Wachstumshemmung bedingt als ein 3%iger Zusatz und die Wachstumshemmung bei den männlichen Tieren prozentual stärker ist als bei den weiblichen Tieren — wahrscheinlich in Zusammenhang mit dem stärkeren Wachstum der männlichen Tiere. Ferner besteht eine deutliche Tendenz zur Abnahme der prozentualen Wachstumshemmung und ihrer Signifikanz gegen Versuchsende, teilweise schon bei den weiblichen Tieren mit 5% Phosphat nachweisbar, regelmäßig aber bei beiden 3%igen Phosphatgruppen beiderlei Geschlechts*). Nach Fütterung mit 1,8% Phosphat besteht nirgends mehr eine Wachstumshemmung. Ob die vorübergehend sogar nachweisbare Wachstumssteigerung nicht irgendwie zufällig zustandegekommen ist, soll durch weitere Versuche geklärt werden. Unterschiede zwischen den 4 Phosphaten in ihrer wachstumshemmenden Wirkung sind nicht deutlich erkennbar. Wenn überhaupt vorhanden, sind sie jedenfalls gering. Es fällt vielleicht auf, daß bei den männlichen Tieren 5% Phosphat das Orthophosphat die am längsten anhaltende Wachstumshemmung bewirkte und daß in der Gruppe mit 5% Phosphat das Grahamsalz die geringste Wirkung zeigte.

Unser Ergebnis steht in guter Übereinstimmung mit den Befunden von Van Esch, Vink, Wit und Van Genderen [3], die mit 5% eine signifikante Wachstumshemmung, nicht mehr aber mit 2,5% erhielten.

Die Letalität schwankte in den Versuchsgruppen zwischen 0 und 6 Tieren. Ein Unterschied zwischen Kontroll- und Phosphattieren ist nicht nachweisbar. Die Erythrocyten und das Hämoglobin zeigten im allgemeinen nur geringe, d. h. unter 10% liegende Schwankungen (Tab. 1). Nur zweimal ging das Ausmaß der Erythrocytensenkung darüber hinaus, nämlich bei den weiblichen Tieren mit 5% Grahamsalz und den männlichen Tieren mit 3% Tripolyphosphat. Die Futteraufnahme war nicht charakteristisch verändert, um als Erklärung für die Wachstumshemmung dienen zu können (Tab. 2). Im allgemeinen war eine gewisse Mehraufnahme, pro kg Körpergewicht gerechnet, bei den mit Phosphat gefütterten Tieren vorhanden, dagegen nicht bei absoluter Berechnung. Die Organgewichte bei den mit 3 und 5% Phosphat gefütterten Tieren zeigten, absolut genommen, meist kein von den Kontrollen abweichendes Verhalten. Vereinzelt kamen allerdings signifikant erhöhte Mittelgewichte bei der Leber und Niere und (am häufigsten) der Milz vor, ohne erkennbaren Zusammenhang mit Art und Konzentration des Phosphats oder mit dem Geschlecht. Thymus, Nebenniere und Herz zeigten vereinzelt sowohl signifikante Zu- als auch Abnahme des Mittelgewichtes, die Lunge einige Male nur eine Abnahme. Die Untersuchung der 1,8%-Phosphattiere steht noch aus.

*) Über ein Teil dieser Ergebnisse wurde von uns (Hahn, F., Jacobi, H. u. Rummel, W., Naturwissenschaften 1956, S. 399) bereits kurz berichtet. Die damalige Angabe, daß 3%iges Grahamsalz, Tripolyphosphat und Pyrophosphat eine signifikante Wachstumshemmung bewirken, bezog sich auf das Ende der Fütterungsperiode.

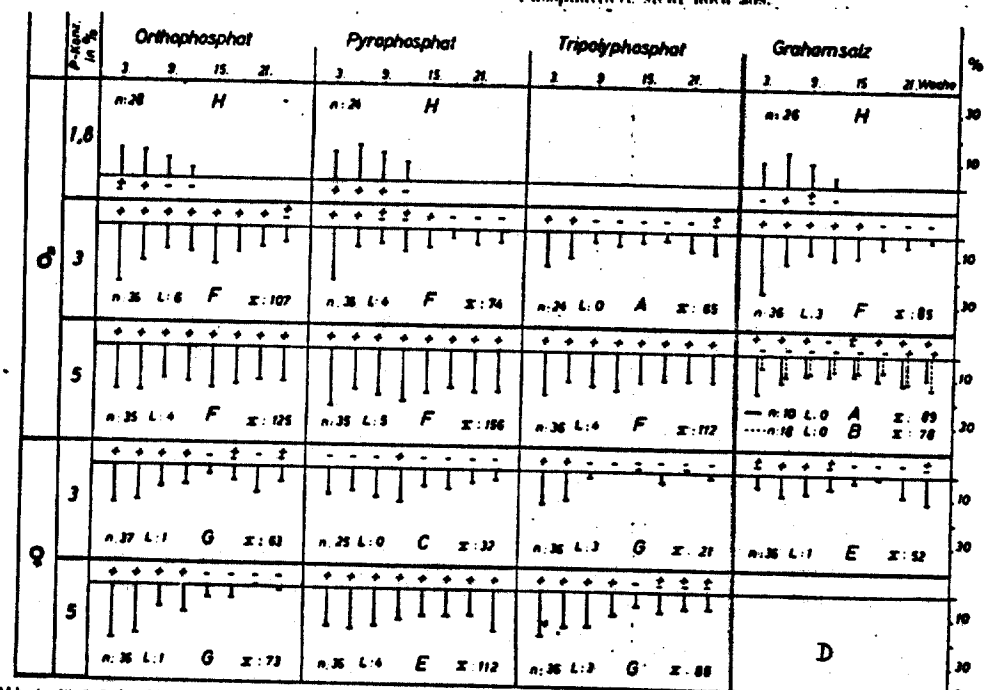

Abb. 1: Einfluß der Phosphate auf das Wachstum. Säulen: Gewichtsdifferenz zwischen Kontroll- und Phosphat-Tieren in % der Kontrolltiergewichte. Säulen nach oben: Phosphat-Tiere überwiegen Kontroll-Tiere; Säulen nach unten: Kontroll-Tiere überwiegen Phosphat-Tiere. Signifikanz: + = p < 0,01; ± = p < 0,02; — = p > 0,02. n = Zahl der Phosphat-Tiere zu Beginn des Versuches. L = Zahl der während des Versuches gestorbenen Phosphat-Tiere. Σ = Summe der Gewichtsdifferenzen. A bis H = zusammengehörige Versuchsabschnitte. Zahl und Letalität der Kontrolltiere: A: n = 25, L = 0; B: n = 17, L = 0; C: n = 25, L = 0; D: n = 36, L = 5; E: n = 36, L = 7; G: n = 36, L = 0; H: n = 36.

Tabelle 1

		Hb %	Erythrocyten in Mill.	Anzahl der Tiere
A Grahamsalz 5%	♂	93 ±2,85	6,23 ±0,279	18
Kontrollen	♂	95 ±3,77	6,36 ±0,278	15
B Tripolyphosphat 5%	♂	93 ±0,52	4,34 ±0,739	24
Grahamsalz 5%	♂	94 ±4,27	4,94 ±0,46	10
Kontrollen	♂	101 ±4,19	5,06 ±0,314	25
C Na-Pyrophosphat 5%	♀	89 ±4,83	4,67 ±0,523	26
Kontrollen	♀	94 ±6,81	4,60 ±0,445	23
D Grahamsalz 5%	♀	88 ±6,06	3,45 ±0,365	24
Kontrollen	♀	89 ±2,51	4,0 ±0,495	14
E Grahamsalz 5%	♀	92 ±4,47	4,73 ±0,307	33
Na-Pyrophosphat 5%	♀	90 ±7,89	4,40 ±0,426	29
Kontrollen	♀	97 ±3,79	4,69 ±0,223	30
F Orthophosphat 5%	♂	92 ±3,71	4,91 ±0,334	30
Na-Pyrophosphat 5%	♂	92 ±6,05	4,61 ±0,509	31
Grahamsalz 5%	♂	93 ±10,8	4,71 ±0,317	33
Orthophosphat 5%	♂	95 ±6,36	4,77 ±0,293	32
Na-Pyrophosphat 5%	♂	85 ±5,97	4,31 ±0,283	29
Tripolyphosphat 5%	♂	85 ±7,85	4,31 ±0,38	30
Kontrollen	♂	93 ±7,7	4,58 ±0,369	29
G Orthophosphat 5%	♀	90 ±7,2	5,33 ±0,434	31
Tripolyphosphat 5%	♀	91 ±11,7	5,77 ±0,652	34
Orthophosphat 5%	♀	93 ±11,9	5,55 ±0,483	29
Tripolyphosphat 5%	♀	88 ±12,7	5,24 ±0,599	33
Kontrollen	♀	93 ±5,9	5,04 ±0,346	33

Phosphor-, Eisen- und Calcium-Gehalt der veraschten, mit Phosphat gefütterten Tiere zeigten keine von den Kontrollen abweichenden Werte, ausgenommen eine signifikante Erhöhung des Ca- und Fe-Gehaltes bei weiblichen Tieren mit 5% Pyrophosphat und des Ca-Gehaltes bei den weiblichen Tieren mit 5% Grahamsalz (Tab. 3).

Der wichtigste histologische Befund ist die von Van Esch, Vink, Witu, Van Genderen [3] beschriebene Neigung zum Auftreten von Kalkniederschlägen, speziell in der Niere.

In Übereinstimmung hiermit fanden auch wir Nierenverkalkungen sowohl bei den mit 5% als auch bei den mit 3% Phosphat gefütterten Tieren. Über das Verhalten der mit 1,8% Phosphat gefütterten Tiere können wir noch nichts aussagen. Die Verkalkungen fehlten bei keinem der 4 Phosphate. Doch war bei den weiblichen Tieren deutlich erkennbar, daß die Verkalkungen bei Grahamsalz (5 und 5%) eine geringere Intensität hatten (Tab. 4). Bei den männlichen Tieren wurde eine ähnliche Beobachtung nach Fütterung mit 3% Grahamsalz gemacht, während in der Gruppe mit der höheren Phosphatkonzentration die Kalkniederschläge aus äußeren Gründen nicht quantitativ ausgewertet wurden.

Tab. 2: Gesamtfutteraufnahme (g/kg) in 6 Monaten.

A Grahamsalz 5%	♂	13 278
Kontrollen	♂	13 039
B Tripolyphosphat 5%	♂	10 828
Grahamsalz 5%	♂	11 498
Kontrollen	♂	9 938
C Na-Pyrophosphat 5%	♀	11 597
Kontrollen	♀	12 067
D Grahamsalz 5%	♀	10 199
Kontrollen	♀	8 927
E Grahamsalz 5%	♀	11 037
Na-Pyrophosphat 5%	♀	11 531
Kontrollen	♀	10 733
F Orthophosphat 5%	♂	12 047
Na-Pyrophosphat 5%	♂	10 719
Grahamsalz 5%	♂	10 616
Orthophosphat 5%	♂	12 971
Na-Pyrophosphat 5%	♂	11 336
Tripolyphosphat 5%	♂	11 600
Kontrollen	♂	10 109
G Orthophosphat 5%	♀	12 641
Tripolyphosphat 5%	♀	13 099
Orthophosphat 5%	♀	12 914
Tripolyphosphat 5%	♀	13 540
Kontrollen	♀	10 252

Tabelle 3

	Geschlecht	n	P	Ca	Fe	Cu
A Kontrollen	♂	15		990 ±120	5,23 ±0,76	0,153 ±0,031
Graham-Salz 5%	♂	17		1010 ±260	5,51 ±0,77	0,178 ±0,042
B Kontrollen	♂	13	299 ±13,1	910 ±50	5,23 ±0,71	0,140 ±0,030
Tripolyphosphat 5%	♂	14	297 ±26,2	910 ±80	5,58 ±0,79	0,145 ±0,016
C Kontrollen	♀	18	323 ±30,7	1070 ±100	5,62 ±0,60	0,177 ±0,026
Na-Pyrophosphat 5%	♀	13	369 ±26,3	980 ±120	5,50 ±1,00	0,181 ±0,043
D Kontrollen	♀	7	332 ±29,4	1110 ±100	4,34 ±1,20	0,145 ±0,014
Graham-Salz 5%	♀	16	325 ±24,1	1010 ±90	4,53 ±1,09	0,159 ±0,025
E Kontrollen	♀	25	346 ±30,5	1130 ±104	5,70 ±0,87	0,234 ±0,027
Na-Pyrophosphat 5%	♀	22	362 ±32,9	1240 ±116	6,49 ±1,22	0,241 ±0,057

Tab. 4: Nierenverkalkungen bei den weiblichen Tieren. Intensitätsgrade: 0, +, ++, +++. Arabische Ziffern: Zahl der Tiere.

	0	+	++	+++	Gesamtzahl der Tiere
Kontrolltiere	7	3			10
3% Orthophosphat			3	7	10
3% Pyrophosphat			4	5	9
3% Tripolyphosphat			4	6	10
3% Grahamsalz	6	3	1		10
5% Orthophosphat			1	9	10
5% Pyrophosphat		1	1	8	10
5% Tripolyphosphat				10	10
5% Grahamsalz	6	3	1		10

Die Nierenveränderungen gehören zum Bild der Kalknephrose (Kalkinfarkt). Es handelt sich dabei um Kalkablagerungen in abgestorbene und abgeschilferte Kanälchenepithelien und um große Kalkschollen, die aus nekrotischen Zellmassen entstanden sind und das Tubuslumen ausfüllen; in fortgeschritteneren Stadien sind die nekrotischen Massen konzentrisch geschichtet. Bei Turnbullblau-Färbung erwiesen sich die Kalkherde als eisenhaltig. Vielfach sind Kanälchenepithelien nekrotisch geworden und in das Lumen abgestoßen, wo sie als Zellzylinder die Kanälchen teilweise ausfüllen. In späteren Stadien sind die Kanälchen teilweise stark erweitert und von einem degenerierenden Wandepithel ausgekleidet, an dem man gelegentlich Mitosen beobachtet. In manchen Fällen kommen größere Eiweißzylinder vor.

Die genannten Veränderungen sind vorwiegend in der inneren Rinde und im äußeren Mark lokalisiert, bei stärkeren Graden der Veränderungen auch in der äußeren Rinde. Die Kalkablagerungen und Epithelnekrosen finden sich hauptsächlich in den distalen Abschnitten der Nephrone: in den Henle'schen Schleifen, in den Tubuli contorti II und in den Sammelröhren. Die mit Grahamsalz gefütterten Tiere zeigten die meist geringgradigen Veränderungen vorwiegend in der inneren Markzone und in der Markpapille.

In allen Gruppen der Versuchstiere kommen kleinere interzelluläre Kalkablagerungen vor. Gelegentlich werden auch Blutfülle, Zellinfiltrate in der Nierenrinde und unter der Nierenkapsel, hyaline und fibröse Herde sowie Plasmazellvermehrungen um die Gefäße beobachtet. Die Nierenkapsel sind im allgemeinen unverändert. Bei einigen Tieren wurden leichte Zellvermehrung, Hyalinisierung und fibröse Verdickung sowie glomerulare Nekrosen mit Wucherung der Bowman'schen Kapsel beobachtet. Diese Veränderungen gehen nicht mit der Art der Fütterung parallel.

Für die Beurteilung der Versuchsgruppen ist das gelegentliche Auftreten von Kalkherden in den Nieren der Kontrollgruppen unerheblich, da in allen Versuchsgruppen Tiere mit deutlich stärkeren Kalkablagerungen als in den Kontrollgruppen vorkommen.

Von den übrigen Organen waren die Lungen bei einigen Tieren stärker verändert. Neben vermehrtem Pigmentgehalt wurden pneumonische Herde mit zellulären entzündlichen Infiltrationen, sowie eitrige Bronchitiden gefunden. Diese Veränderungen dürften mit den Versuchsbedingungen nicht in Zusammenhang stehen. Am Herzen kommen bei einzelnen Tieren wenige feinste Zellinfiltrate vor; die Lebern haben vakuolare Zellveränderungen bei Versuchs- und Kontrolltieren. In der Milz fällt eine wechselnde Menge von Pigmenten auf. In einigen Fällen findet man Riesenzellen. Thymus, Nebenniere, Dickdarm und Dünndarm sind unverändert.

Besprechung der Ergebnisse:

Unsere Versuche geben auf die eingangs gestellte Frage, ob Polyphosphate toxikologisch ernster zu bewerten sind als das Orthophosphat, eine eindeutige Antwort. Polyphosphate sind, wenn das Wort „giftig", das ja nur ein Quantitätsbegriff ist, in diesem Zusammenhang einmal gebraucht werden darf, bestimmt nicht giftiger als das „natürliche" Orthophosphat. Eher ist das Umgekehrte der Fall, wenn auch der Unterschied in dieser Richtung nur gering ist und sich daher leicht dem Nachweis entzieht. Es ist aber möglich, daß dieser Unterschied größer wird, wenn durch Veränderung der Versuchsbedingungen oder der Tierart die Spaltungsbedingungen für Polyphosphate im Darm verändert werden können. Die weitgehende quantitative und qualitative Ähnlichkeit in den toxikologischen Eigenschaften der 4 geprüften Phosphate spricht dafür, daß sie nicht anders als nach ihrem Gehalt an Orthophosphat und ihrer Spaltbarkeit in Orthophosphat toxikologisch zu werten sind.

Die Ursache der Wachstumshemmung durch höchste Konzentrationen (3–5%) ist noch unbekannt. Vielleicht handelt es sich bei der peroralen Zufuhr der Phosphate überhaupt nur um osmotische Effekte (Zipf [9]). Ob ein Zusammenhang mit den Verkalkungen in der Niere besteht, ist fraglich, zumal diese auch bei noch niederen, nicht mehr wachstumshemmenden Konzentrationen vorkommen (van Esch, Vink, Wit u. van Genderen [3]). Der Entstehung dieser Verkalkungen und ihrer Reversibilität nachzugehen, ist das Ziel unserer weiteren Untersuchungen. Die Möglichkeit einer Störung der Resorption lebenswichtiger Elemente erfährt durch das Verhalten des Blutbildes und den Veraschungsanalysen keine Stütze.

Zusammenfassung:

1. Es wird über chronische Fütterungsversuche mit verschiedenen Kondensationsstufen der Orthophosphorsäure (= Monophosphorsäure), wie Ortho-(= Mono-)-phosphat, Pyro-(= Di-)-phosphat, Tripoly-(= Tri-)-phosphat und Grahamsalz an Ratten berichtet.
2. Die vier Phosphate unterscheiden sich quantitativ und qualitativ in ihrer Wirkung nicht wesentlich. 3- und 5%ige Phosphatzusätze zur Nahrung (Sherman-Diät) bewirken eine signifikante Wachstumshemmung, die bei 3% Phosphat nur vorübergehend ist, bei 5% Phosphat bei einer Versuchsdauer von 6 Monaten meist bis zum Schluß vorhanden ist. 1,8%ige Zusätze wirken nicht mehr wachstumshemmend.
3. Die Untersuchungen des roten Blutbildes, die Bestimmung der Organgewichte und die Veraschungsanalyse der Gesamttiere auf P, Ca, Fe und Ca deckten keine charakteristischen Veränderungen auf.
4. Typisch sind Veränderungen der Niere mit Verkalkungen, die bisher von uns bei allen vier Phosphaten noch bei 5%iger Konzentration in der Nahrung nachgewiesen wurden. Die Verkalkungen waren bei den Tieren, die Grahamsalz erhalten hatten, geringer als bei den mit anderen Phosphaten gefütterten Tieren.

Summary:

Different Compatability of Orthophosphate and Polyphosphates in Chronic Feeding Tests?

1. Report on chronic feeding tests with different stages of condensation of orthophosphoric acid (= monophosphoric acid), for instance ortho-(= mono)phosphate, pyro-(= di)phosphate, tripoly-(= tri)phosphate and Graham's salt in rats.
2. The four phosphates show no marked quantitative or qualitative difference of action. Significant inhibition of growth is induced by adding 3% and 5% phosphates to the food (Sherman diet) which is transient in 3% phosphate, while it may still be observed after a duration of investigation of 6 months in 5% phosphates. No inhibition of growth is induced by adding 1,8% phosphates to the food.
3. Investigations of the red blood count, determination of the weight of organs and ash analysis of the total test animals for P, Ca, Fe and Ca showed no significant changes.
4. Typical changes in the form of calcification are observed in the kidneys following 3% concentrations of all four phosphates in the food. Calcifications were less marked in animals which had been treated with Graham's salt than in those treated with other phosphates.

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Eine neue Methode zur quantitativen Auswertung von Rundfilter-Chromatogrammen durch indirekte Densitometrie

(Aminosäuren, Glykoside der *Convallaria maj.*)

Von Professor Dr. H. Erbring, Dr. P. Patt und N. Frey

Die quantitative Bestimmung von Substanzgemischen nach papierchromatographischer Auftrennung ist im Schrifttum wiederholt vorgeschlagen und kritisch diskutiert worden [1]. Hierbei handelt es sich im wesentlichen um die Auswertung von ein- oder zweidimensionalen Chromatogrammen. Während der letzten Jahre hat sich die Rundfilter-Methodik auf Grund der Erzielung besserer Trennschärfen zur Lösung spezieller Fragestellungen immer mehr in den Vordergrund geschoben. Unseres Wissens sind jedoch bisher keine geeigneten Vorschläge zur direkten quantitativen Auswertung dieser Chromatogramme gemacht worden. Der Grund liegt zweifellos in der besonderen Schwierigkeit, den Flächenzuwachs mit steigendem Rf-Wert zu berücksichtigen. Bei dem von Erbring u. Patt [2] vorgeschlagenen Verfahren zur quantitativen Bestimmung von Ascorbinsäure auf dem Wege der Trennung durch Rundfilter-Chromatographie der Ausgangssubstrate handelt es sich um ein modifiziertes Elutionsverfahren, nicht jedoch um eine direkte Auswertung des Chromatogramms. Es war daher wünschenswert, ein Verfahren zu finden, welches dieser letzteren Zielsetzung Rechnung trägt.

Wir haben eine entsprechende Methodik ausgearbeitet, der die densitometrische Ausmessung der einzelnen Substanz-Zonen zugrunde liegt. Es war naheliegend, dieses Verfahren zunächst am Beispiel der bereits umfassend untersuchten Aminosäuren sicherzustellen, um es auf andere, ähnlich gebaute Naturstoffe zu übertragen.

Rundfilter-Chromatographie

Die von uns verwendete Anordnung zur Durchführung der Rundfilter-Chromatographie geht aus den Abb. 1 u. 2 hervor. Die Pipette enthält das Entwicklungsmittel, und zwar für Aminosäuren: Butanol-Eisessig-Wasser 4 : 1 : 1; für Herzglykoside der *Convallaria maj.*: Essigester, wassergesättigt. Der Essigester muß frisch destilliert und zur Entfernung etwaiger Essigsäure zu-

Rocznik: Państw. Zakł. Hig. 9: 441-9 (1958)

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IRENA JAMPOLER, STEFAN KRUŚ

NIEKTÓRE ZAGADNIENIA DOTYCZĄCE POWSTAWANIA DOŚWIADCZALNEJ MARTWICY WĄTROBY U SZCZURÓW NA TLE NIEDOBORÓW POKARMOWYCH

III. DZIAŁANIE OCHRONNE FOSFORANU POTASU ZAWARTEGO W DIECIE MARTWICOTWÓRCZEJ

Z Zakładu Higieny Żywienia PZH i z Zakładu Patomorfologii PAN

W licznych badaniach dotyczących martwicy wątroby u szczurów na tle niedoborów pokarmowych (1) rozpatrywano znaczenie i działanie rozmaitych składników diety. Okazuje się, że do tej pory nie zbadano jeszcze roli zawartych w diecie soli mineralnych. Gillman i współpr. (2), którzy stosowali bardzo prostą dietę martwicotwórczą, złożoną z drożdży i skrobi ziemniaczanej, spostrzegli, iż dodanie do takiej diety mieszaniny soli mineralnych opóźnia występowanie martwicy wątroby. Autorzy ci ograniczyli się jednak tylko do stwierdzenia tego zjawiska i nie opracowali go dokładniej. Wykonane przez nas badania orientacyjne pozwoliły nam przypuszczać, że sprawa ta jest godna bliższego zainteresowania. Dlatego postanowiliśmy zbadać wpływ niektórych soli mineralnych na powstawanie martwicy wątroby.

A. Zawartość Na, K, Ca i P w różnych drożdżach a stopień ich działania martwicotwórczego.

Pracę rozpoczęliśmy od oznaczenia poziomu Na, K, Ca i P w próbkach drożdży i mączki łubinowej, użytych przez nas w jednej z poprzednich prac (3), a także w próbkach drożdży użytych w bieżących doświadczeniach.* Wyniki oznaczeń przedstawia tabela I.

Jak widać zawartość jonów Na, K i Ca w różnych gatunkach drożdży jest zbliżona; dlatego wydaje się, że nie można z nią wiązać różnego stopnia działania martwicotwórczego tych drożdży. Zwraca natomiast uwagę duża zawartość fosforu w mało martwicotwórczych drożdżach piwnych.

B. Doświadczenia biologiczne.

W doświadczeniach zastosowaliśmy 2 typy diety martwicotwórczej:

a) z drożdżami piwnymi,

b) z drożdżami *Torula utilis*.

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* Oznaczenia Na, K i Ca za pomocą spektrografii płomieniowej wykonał ob. J. Widła z Zakładu Fizyki Technicznej Instytutu Chemii Ogólnej (kierownik: doc. mgr J. Świętosławska). Składamy Mu na tym miejscu serdeczne podziękowanie.

Skład diet:
drożdże piwne 16,5% albo drożdże *Torula utilis* 20,6%,
sole mineralne (U.S.P., nr 2, XIII, str. 72, 1947) 3%,
smalec 6%,
tran 1%,
skrobia pszenna do 100%.

Drożdże piwne i *Torula utilis* różnią się między sobą zawartością białka (60,7% i 48,5%). Aby ilość białka w obu dietach była jednakowa (9%), musieliśmy użyć różnych ilości drożdży.

Tabela I

Surowiec	Działanie martwicotwórcze	Na	K	Ca	P
		% suchej substancji			
Drożdże *Torula utilis* z poprzedniej pracy (4)	silne	0,2	2,62	0,4	0,83
Drożdże *Torula utilis* z pracy bieżącej		0,21	2,62	0,3	0,74
Drożdże piwne z pracy poprzedniej (4)	słabe	0,18	1,90	0,2	1,77
Drożdże piwne z pracy bieżącej		0,18	1,65	0,2	1,53
Drożdże piekarniane z pracy poprzedniej (4)	silne	0,27	2,86	0,4	0,57
Mączka łubinowa	najsłabsze	0,16	1,15	0,4	0,22

DOŚWIADCZENIE 1

Skład mieszaniny soli mineralnych a działanie martwicotwórcze diety.

W doświadczeniu tym podawaliśmy różnym grupom szczurów tę samą dietę martwicotwórczą, zmieniając jedynie skład mieszaniny soli mineralnych (tabela II). Źródłem białka w diecie były drożdże piwne.

Mieszaniny soli mineralnych.

Ustalając skład mieszanin wychodziliśmy z założenia, że drożdże jako pasza dla zwierząt zawierają stosunkowo za dużo fosforu i potasu, a za mało sodu i wapnia. Wobec tego wzbogaciliśmy mieszaninę soli w te jony, które — jak sądziliśmy — znajdują się w diecie z drożdżami w zbyt małej ilości. W ten sposób staraliśmy się doprowadzić do prawidłowego stosunku Na:K i Ca:P. Prawidłowy stosunek Na:K w diecie dla szczurów powinien wynosić 1:3, a nawet 1:1; prawidłowy stosunek Ca:P wynosi od 1:1,5 do 1:1 (4). Uwzględniając fakt, że stosunek Na:K drożdżach wynosi ok. 1:10, a stosunek Ca:P ok. 1:4 opracowaliśmy typów mieszaniny soli mineralnych:

mieszanina 1 (kontrolna) — zwykła mieszanina soli (U.S.P. nr 2),

mieszanina 2 — poprawienie stosunku sodu do potasu przez zwiększenie ilości chlorku sodu,

mieszanina 3 — poprawienie stosunku sodu do potasu i wapnia do fosforu przez pominięcie K_2HPO_4, (obniżenie poziomu potasu i fosforu),

mieszanina 4 — obniżenie poziomu sodu, potasu i fosforu,

mieszanina 5 — poprawienie stosunku sodu do potasu przez pominięcie źródła potasu; odpowiednią ilość fosforu dodano pod postacią fosforanu wapnia,

mieszanina 6 — obniżenie ilości fosforu przez pominięcie fosforanu potasu i podanie odpowiedniej ilości potasu pod postacią chlorku potasu.

Skład tych 6 mieszanin soli podano w tabeli II. Pominięte składniki mineralne zastąpiono skrobią pszenną. Z przyczyn technicznych fosforan potasu obojętny $KaPO_4$ z mieszaniny podstawowej zastąpiliśmy przez fosforan potasu dwurzędowy K_2HPO_4.

Tabela II

Mieszanina soli mineralnych	Składniki mieszaniny soli mineralnych w g									Łącznie w g
	Na Cl	$Na_2HPO_4 \cdot 7H_2O$	K_2HPO_4	$CaHPO_4 \cdot 2H_2O$	$CaC_2H_2O_4 \cdot 5H_2O$	$MgSO_4 \cdot 7H_2O$	cytrynian Fe . XH_2O	KCl	skrobia pszenna	
1. USP nr. 2	1,73	3,47	11,72	5,40	13,00	5,45	1,18	—	—	41,95
2.	8,97	3,47	11,72	5,40	6,06	5,45	1,18	—	—	41,95
3.	1,73	3,47	—	5,40	13,00	5,45	1,18	—	11,72	41,95
4.	—	—	—	5,40	13,00	5,45	1,18	—	16,92	41,95
5.	1,73	3,47	—	16,95	—	5,45	1,18	—	13,17	41,95
6.	1,73	3,47	—	5,40	13,00	5,45	1,18	9,90	1,82	41,95

Opisane mieszaniny soli mineralnych włączono do 6 diet z drożdżami piwnymi o jednakowym poza tym składzie. Diety te zastosowano u 6 grup szczurów hodowlanych, dobranych wiekiem i wagą; każda grupa składała się z 8 zwierząt (4 samce i 4 samice). Warunki wykonywania doświadczenia były takie same jak w naszych pracach poprzednich (3, 5). Codziennie oznaczano ilość pokarmu zjadanego przez szczury. Okazało się, że szczury zjadały przeciętnie dziennie po ok. 12 g pokarmu, zawierającego 16,5%, tzn. 2 g drożdży piwnych i 3% (0,36 g) soli mineralnych. Pozwoliło to nam na wyliczenie, ile Na, K, Ca i P zjadał dziennie szczur wraz z dietą (tabela III).

W toku doświadczenia szczury padały z powodu martwicy wątroby. Zwierzęta padłe sekcjonowano, z wątroby pobierano wycinki i utrwalano je w formalinie. W wątrobach tych stwierdzaliśmy zawsze obrazy mikroskopowe typowe dla martwicy (1). Szczury pozostałe przy życiu zabito po 63 dniach — w wątrobach ich nie stwierdzono odchyleń od stanu prawidłowego.

Miarą działania martwicotwórczego każdej diety jest przeciętny okres przeżycia szczurów i śmiertelność, tj. stosunek liczby zwierząt padłych wskutek martwicy wątroby do całkowitej liczby zwierząt w danej grupie. Wartości te podano w tabeli IV.

Tabela III

2 g drożdży piwnych zawierają: Na — 3,56 mg, K — 32,60 mg, Ca — 3,96 mg, P — 30,50 mg

Grupa	Pierwiastek	Zawartość w 0,36 g mieszaniny soli mineralnych w mg	Zawartość w 12 g diety w mg	Na : K Ca : P	Grupa	Pierwiastek	Zawartość w 0,36 g mieszaniny soli mineralnych w mg	Zawartość w 12 g diety w mg	Na : K Ca : P
1	Na	10,68	14,24	1 : 5	4	Na	—	3,56	1 : 9
	K	45,50	78,10			K	—	32,60	
	Ca	25,40	29,36	1 : 2		Ca	25,40	29,36	1 : 1,3
	P	29,70	60,20			P	8,31	38,81	
2	Na	34,22	37,78	1 : 2	5	Na	10,68	14,24	1 2
	K	45,50	78,10			K	—	32,60	
	Ca	17,62	21,58	1 : 3		Ca	34,00	37,96	1 : 1,5
	P	29,70	60,20			P	29,70	60,20	
3	Na	10,68	14,24	1 : 2	6	Na	10,68	14,24	1 : 5
	K	—	32,60			K	45,50	78,10	
	Ca	25,40	29,36	1 : 1,5		Ca	25,40	29,36	1 : 1,5
	P	11,75	42,25			P	11,75	42,25	

Tabela IV

Grupa	Mieszanina soli mineralnych	Przeciętna waga początkowa w g	Przeciętne spożycie dzienne w g	Przeciętny okres przeżycia w dniach	Śmiertelność z powodu martwicy wątroby w ciągu 63 dni
1	1 (zawiera K_2HPO_4)	62,5	12,5	1 szczur padł po 29 dniach	1/8
2	2 (zawiera K_2HPO_4)	62,1	11,6	23	3/8
3	3 (nie zawiera K_2HPO_4)	62,0	13,0	33,3	8/8
4	4 (nie zawiera K_2HPO_4)	62,5	10,9	30,7	8/8
5	5 (nie zawiera K_2HPO_4)	61,5	10,9	26,0	7,8
6	6 (nie zawiera K_2HPO_4)	61,3	11,6	31,7	8,8

Omówien
pierwszej wynos
wszystkie lub pr
więc niską śmier
tego faktu rozpat
się od siebie i ja
wszystkim zwróc
siebie (tabela III)
się natomiast, że
grupy 3—6 był i
1 i 2 otrzymywał
ciliśmy uwagę w
ralnych o stałym

Sprawdz
foranu pot
W doświadczen
i 4 samice). Gru
piwnych, grupy
grupy 5 i 6 były
Mieszaninę soli m
w grupie 1 i 7
w grupie 2 i 8
jaka znajduje się
w grupie 3 i 9
sunku do grupy k
w grupie 4 i 1
sunku do diety k
Skład mieszani

Grupy szczurów	Mieszanina soli mineralnych
1, 5, 7	1.
2, 6, 8	2. USP nr 2
3, 9	3.
4, 10	4.

Doświadczenie
Szczury padłe se
histologicznego. W

Omówienie wyników. Liczba zwierząt padłych w grupie pierwszej wynosi 1, w grupie drugiej — 3, w grupie 3, 4, 5 i 6 padły wszystkie lub prawie wszystkie szczury. Grupy 1 i 2 charakteryzują się więc niską śmiertelnością, a grupy 3—6 wysoką. Szukając wyjaśnienia tego faktu rozpatrzyliśmy, pod jakim względem te dwie grupy diet różnią się od siebie i jaka cecha powtarza się w obrębie każdej z nich. Przede wszystkim zwróciliśmy uwagę na poszczególne jony i ich stosunek do siebie (tabela III) i nie znaleźliśmy tutaj żadnej prawidłowości. Okazało się natomiast, że najwyraźniejszą wspólną cechą diety spożywanej przez grupy 3—6 był brak fosforanu potasu dwurzędowego; natomiast grupa 1 i 2 otrzymywała tę sól. Wobec tego w następnym doświadczeniu zwróciliśmy uwagę wyłącznie na fosforan potasu i w mieszaninie soli mineralnych o stałym składzie sól ta była jedyną zmienną.

DOŚWIADCZENIE 2

Sprawdzenie wpływu różnych ilości fosforanu potasu w diecie martwicotwórczej.

W doświadczeniu tym utworzyliśmy grupy po 8 szczurów (4 samce i 4 samice). Grupy 1—4 otrzymywały dietę zawierającą 16,5% drożdży piwnych, grupy 7—10 dietę zawierającą 20,6% drożdży *Torula utilis*; grupy 5 i 6 były powtórzeniem grup 1 i 2 na większej liczbie szczurów. Mieszaninę soli mineralnych dobraliśmy w ten sposób, aby:

w grupie 1 i 7 nie zawierała fosforanu potasu,

w grupie 2 i 8 (kontrolnych) zawierała tę ilość fosforanu potasu, jaka znajduje się zwykle w mieszaninie kontrolnej (USP, nr 2),

w grupie 3 i 9 zawierała podwojoną ilość fosforanu potasu w stosunku do grupy kontrolnej,

w grupie 4 i 10 zawierała potrojoną ilość fosforanu potasu w stosunku do diety kontrolnej.

Skład mieszanin soli mineralnych przedstawiono w tabeli V.

Tabela V

Grupy szczurów	Mieszanina soli mineralnych	Składniki mieszaniny soli mineralnych wg								Łącznie w g	% mieszaniny soli w diecie
		Na Cl	$Na_2HPO_4 \cdot 7H_2O$	K_2HPO_4	$CaHPO_4 \cdot 2H_2O$	$Ca(C_6H_5O_7)_2 \cdot 5H_2O$	$MgSO_4 \cdot 7H_2O$	cytrynian Fe $\cdot XH_2O$	skrobia pszenna		
1, 5, 7	1.	1,73	3,47	—	5,40	13,00	5,45	1,18	11,72	41,95	3,00
2, 6, 8	2. USP nr 2	1,73	3,47	11.72	5,40	13,00	5,45	1,18	—	41,85	3,00
3, 9	3.	1,73	3 47	23.44	5,40	13,00	5,45	1,18	—	53,67	3.84
4, 10	4.	1,73	3,47	35,16	5,40	13,00	5,45	1,18	—	65,39	4,68

Doświadczenie to wykonano podobnie jak doświadczenie poprzednie. Szczury padłe sekcjonowano, z wątroby pobierano wycinki do badania histologicznego. W większości przypadków stwierdzono pod mikrosko-

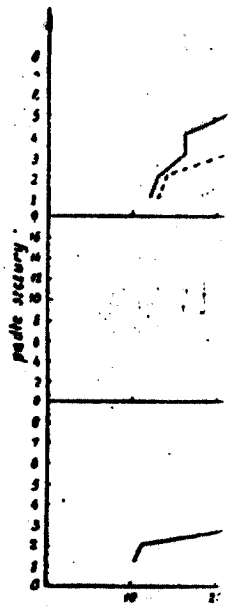
pem martwicę wątroby. W doświadczeniu tym po raz pierwszy widywaliśmy także zwierzęta padłe nie z powodu martwicy wątroby. Szczury, które przeżyły, zabito po 80 dniach. W wątrobach ich nie stwierdzono odchyleń od stanu normalnego.

Doświadczenie to, którego wyniki podajemy w tabeli VI, potwierdziło wyraźnie istnienie ochronnego wpływu fosforanu potasu, wyrażającego się zmniejszeniem śmiertelności w przypadku diety z drożdżami piwnymi i przedłużeniem okresu przeżycia w przypadku diety z drożdżami *Torula utilis*.

Pomimo prawidłowości wyników postanowiliśmy powtórzyć część doświadczenia na większej liczbie szczurów. Powtórzono zatem grupę 1 i 2 (w tablicy VI oznaczone liczbą 5 i 6), używając po 16 szczurów (8 samic i 8 samców). Doświadczenie trwało 149 dni. Wyniki dotyczące wszystkich grup od 1 do 10 zestawiliśmy w tabeli VI. Przebieg doświadczenia, wyrażający się liczbą szczurów padłych z powodu martwicy wątroby w poszczególnych dniach doświadczenia, przedstawia wykres 1.

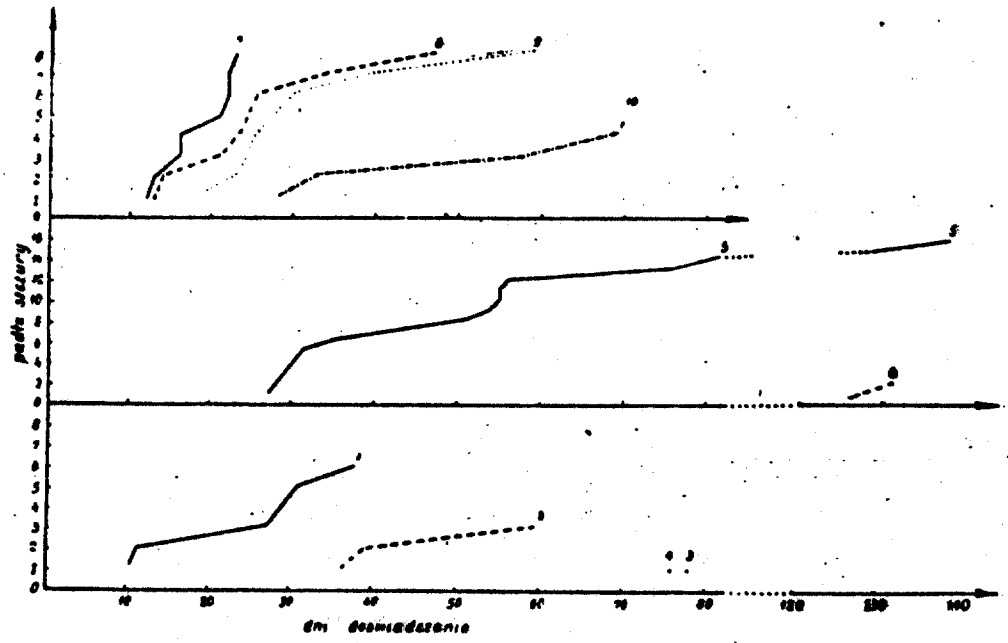
Tabela VI

Grupa	Rodzaj drożdży w diecie	Ilość K_2HPO_4 w mg		Przeciętne spożycie dzienne diety w g	Przeciętna waga szczura w g		Przeciętny okres przeżycia szczurów padłych skutkiem martwicy wątroby w dniach	Śmiertelność skutkiem martwicy wątroby	Śmiertelność z innych przyczyn
		na 100g diety	przeciętnie spożyta dziennie w przeliczeniu na 100 g wagi szczura		początkowa	w toku doświadczenia			
								w ciągu 80 dni	
1	piwne	—	—	14,1	58,0	69,1	24,3	6/7	—
2	„	838	134	12,8	58,0	80,4	44,6	3/8	—
3	„	1 676	250	12,4	60,0	83,3	1 szczur padł po 78 dniach	1/8	—
4	„	2 514	390	10,5	57,0	67,1	1 szczur padł po 76 dniach	1/8	2/8
								w ciągu 149 dni	
5	piwne	—	—	11,1	81,9	99,9	57,3	16/16	—
6	„	838	87	11,7	82,0	112,5	2 szczury padły po 128 dniach	2/16	—
								w ciągu 80 dni	
7	*Torula utilis*	—	—	11,0	73,8	76,5	18,1	8/8	—
8	„	838	115	10,8	73,0	78,7	25,1	8/8	—
9	„	1 676	241	10,8	69,4	75,1	30,8	8/8	—
10	„	2 514	308	9,1	73,2	75,0	51,6	8/8	2/8



Za pow
2 z wyró
W doświadc
kontrolne grup
życie mogłoby
twicy, powtórz
spożycie grupy
szczury grupy
my w tabeli
w obu grupach
działaniem fos

Grupa	Rodzaj drożdży w diecie
8a	*Torula utilis*
10a	„



Za powtórzenie części doświadczenia 2 z wyrównaniem spożycia.

W doświadczeniu 2 szczury grupy 10 żyły 2 razy dłużej niż szczury kontrolne grupy 8, ale jadły mniej pokarmu. Ponieważ zmniejszone spożycie mogłoby samo przez się wpłynąć na opóźnienie powstawania martwicy, powtórzyliśmy tę część doświadczenia (grupa 8 i 10), ograniczając spożycie grupy kontrolnej (8a) do tej ilości, jaką spożywały bliźniacze szczury grupy 10a. Doświadczenie to trwało 42 dni, a wyniki jego podajemy w tabeli VII. Widać z nich, że także przy wyrównanym spożyciu w obu grupach różnica śmiertelności wyraźnie przemawia za ochronnym działaniem fosforanu potasu.

Tabela VII

Grupa	Rodzaj drożdży w diecie	Ilość K_2HPO_4 w mg: na 100 g diety	Ilość K_2HPO_4 w mg: przeciętnie spożyta dziennie w przeliczeniu na 100 g wagi szczura	Przeciętne spożycie dzienne diety w g	Przeciętna waga szczura w g: początkowa	Przeciętna waga szczura w g: w toku doświadczenia	Przeciętny okres przeżycie szczurów padłych skutkiem martwicy wątroby w dniach	Śmiertelność skutkiem martwicy wątroby	Śmiertelność z innych przyczyn
								W ciągu 42 dni	
8a	*Torula utilis*	838	95,6	6,8	57,0	59,8	25,2	8/8	—
10a	„	2 514	262	6,9	57,0	66,1	1 szczur padł 21 dnia	1/8	1/8

OCENA WYNIKÓW I WNIOSKI

1. Skład mineralny diety martwicotwórczej wpływa na intensywność jej działania.

2. Dodanie fosforanu potasu do diety martwicotwórczej osłabia jej działanie. Wpływ ochronny dodanego do diety z drożdżami piwnymi fosforanu potasu wyraża się zmniejszeniem liczby szczurów padłych z powodu martwicy wątroby (grupy 1—4 i 5—6), natomiast w przypadku drożdży *Torula utilis* polega on przede wszystkim na przedłużeniu okresu przeżycia. Przy dostatecznie dużej dawce fosforanu potasu różnica okresów przeżycia szczurów otrzymujących i nie otrzymujących tej soli staje się znamienna statystycznie. Widać to wyraźnie już przy porównaniu grupy 7 i 9 z zastosowaniem próby „t" Studenta (6) ($t=2{,}62$, $k=14$, $P=0{,}021$).

W warunkach wyrównanego spożycia również ujawnia się działanie ochronne fosforanu potasu (grupy 8a i 10a).

3. Dotychczasowe doświadczenia wskazują na to, że działanie ochraniające fosforanu potasu jest proporcjonalne do jego ilości w diecie. Po przekroczeniu jednak pewnego poziomu fosforanu potasu (ok. 250 mg na 100 g wagi szczura) spostrzegliśmy objawy szkodliwego działania ubocznego, mianowicie pojedyncze szczury traciły na wadze i ginęły z wyniszczenia. U szczurów tych nie stwierdziliśmy mikroskopowo zmian w wątrobie.

4. Według Chernicka, Moe, Rodnana i Schwarza (7) uchwytne zaburzenie zjawisk biochemicznych w wątrobie jeszcze przed wystąpieniem martwicy wyraża się obniżeniem zużycia tlenu przez skrawki wątrób szczurów na diecie martwicotwórczej; dodanie dwu-fosfo-nukleotydu powoduje pewne nasilenie oddychania. Autorzy wysnuli przypuszczenie, że obniżenie oddychania jest wykładnikiem zaburzenia fosforylacji oksydatywnej. Łącząc wyniki naszego doświadczenia z poglądem wspomnianych autorów przypuszczamy, że jony fosforanu potasu włączają się w łańcuch reakcji tego procesu, czyli fosforylacji oksydatywnej. W związku z tym spostrzeżenie nasze może być przyczynkiem do poznania mechanizmu powstawania martwicy wątroby na tle niedoborów pokarmowych.

И. Ямполер, С. Крусь

НЕКОТОРЫЕ ВОПРОСЫ ОТНОСЯЩИЕСЯ К ВОЗНИКНОВЕНИЮ ЭКСПЕРИМЕНТАЛЬНОГО НЕКРОЗА ПЕЧЕНИ У КРЫС НА ФОНЕ КОРМОВЫХ НЕДОБОРОВ

III. Защитное действие фосфорнокислого калия находящегося в некрозотворной диете

Содержание

Установлено, что минеральный состав диеты влияет на ее некрозотворное действие. Фосфорнокислый калий добавляемый к некрозотворной диете оказывает защитное действие, заключающееся в статистически знаменательным продолжении среднего периода переживания крыс и уменьшении смертности.

По мере увеличивания дозы фосфорнокислого калия защитное действие возрастало, но после превышения количества около 250 мг на 100 г веса крысы являлись нежелательные побочные симптомы.

Некоторые авторы предполагают, что существенным расстройством перемен печени крысы на некрозотворной диете является расстройство оксидирующей фосфорилизации; быть может, что защитное действие фосфорнокислого калия состоит в том, что его ионы включаются в цепь реакции этого процесса.

I. Jampoler, S. Kruś

SELECTED PROBLEMS OF EXPERIMENTAL DIETARY LIVER NECROSIS IN RATS

III. Protective effect of potassium phosphate added to a necrogenic diet

Summary

It was shown that the mineral composition of a diet influences its necrogenic activity.

The addition of potassium phosphate to a necrogenic diet has a protective effect causing a significant increase of the average survival time of the rats and a decrease of mortality.

The increase of the dosis improves the protective effect; however doses above 250 mg per 100 g of rat body weight produce secondary ill-effects.

Some authors suppose that the cause of this type of liver necrosis lies in defective oxydative phosphorylation; it is possible that the protective action of potassium phosphate is due to its ions being involved in this process.

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J. Amer. Water Works Assoc. 32: 1471-83 (1940)

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The Physiology of Sodium Hexametaphosphate

By K. K. Jones

THE participation of the phosphates in the functions of the cells and the processes of life has been known for a hundred years or more, but a true conception of the fundamental nature of these reactions has been perceived only in the last decade; today we are merely at the threshold of a true understanding of their nature. Known as a chemical curiosity for one hundred years, sodium hexametaphosphate* has but recently been rediscovered to have unique properties that make it of industrial importance and that have suggested far-reaching physiological possibilities.

In order to discuss these possibilities it is important to see the metaphosphates in relation to the activity of the phosphates as a general group in the body. These are found wherever fundamental exchanges of matter and energy take place in the organism. It is interesting to see how the phosphates became so involved in the life of the cell and for this reason let us examine briefly the chemical development of the organism. In broad outline, without attention to details, scientists like Lotka (1), Macallum (2), and others, tell us that life undoubtedly began in the sea, eons ago, when it was not as salty as it is today. The earth at that time was warmer than it is now, and the sky was filled with water vapor. Tremendous electric storms and torrential rains were almost continuous. The atmosphere in these early times contained much more carbon dioxide than it does at this time.

This carbon dioxide dissolved in the water to form carbonic acid, H_2CO_3. The actinic energy of the sun drove two atoms of oxygen

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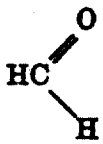
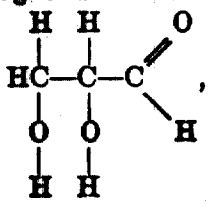
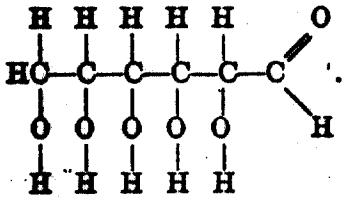
A paper presented on April 24, 1940, at the Kansas City Convention by K. K. Jones, Assistant Professor of Physiology and Pharmacology, Northwestern University Medical School, Chicago.

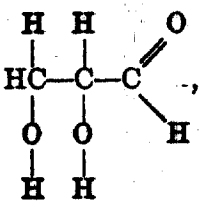
* Hereinafter referred to as hexametaphosphate.

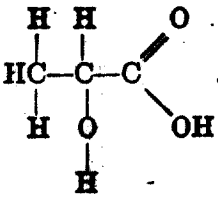
Metaphos

from this molecule, forming formaldehyde,  . In the presence of magnesium salts this formaldehyde condensed to glyceric aldehyde,  , and to hexose and pentose sugar,

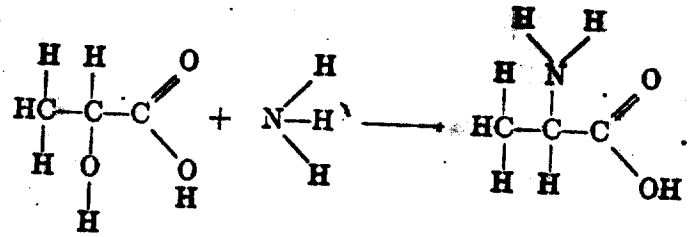
 .

Not only could formaldehyde form sugars, but it could condense to aldehyde and aldol and this, in turn, to long carbon chains which lost oxygen and formed fatty acids.

Glyceric aldehyde,  , readily rearranged its atoms

and became lactic acid,  .

In the atmosphere the electrical discharges of lightning synthesized ammonia from the atmospheric nitrogen and water, and this ammonia in combination with formaldehyde, through similar condensation, formed the amino acids. For example, the amino acid, alanine, could be formed from lactic acid and ammonia.



With amino acids, fatty acids, and sugars, Nature had building material for the formation of proteins, fats, and carbohydrates, and could begin the process of building protoplasm. Of the intricacies of building protoplasm we know very little, but what we do know indicates that the inorganic ions in the sea water played a fundamental role.

In the same way as magnesium was able to effect a synthesis of sugar, so the other elements in the sea, sodium, potassium, calcium, iron, chlorine, and phosphorus each took part in the formation of protoplasm and became intimately associated with life processes. In fact, many of the synthesized organic compounds were able to combine with different elements or ionic radicals, so that a competition arose between the ions in this regard. Particularly was this true between the active potassium ions and the far more numerous sodium ions. It may even be said that the formation of the cell was an expression of the attempt of the potassium compounds to protect themselves from domination by the sodium ions.

In this struggle for existence the important compounds were the protein molecules. As these various protein molecules grew in complexity, they began to compete for the various amino acids and prosthetic groups from which they could construct their molecules. Out of this competition arose the ability of one protein aggregate to attack and break down another in order to utilize its constituent parts for building material. In other words, enzymes developed.

The calcium ion was utilized by these protein aggregates for its ability to form emulsions with fats that were immiscible in water and that, therefore, could be used to protect the newly forming protoplasm.

For this reason calcium is usually localized in the limiting membranes of the cell and the organism. Iron, because of its great reactivity with oxygen became an intermediary for oxygen transport and found its place in every cell.

Functions of the Phosphate Ion

Around each inorganic ion or radical, therefore, there centered various activities which were peculiar to the chemical characteristics and properties of these elements. Each one is a story in itself, but of all these ions, none assumed a more fundamental role or achieved greater importance than the phosphate ion. We cannot trace the development of these activities but we can list many of the functions:

1. The tri-basic nature of orthophosphoric acid made it possible for mono- and di-basic phosphate salts to be formed. Mixtures of these salts in solution had the property of resisting any change that might take place in the hydrogen-ion concentration of the water in which they were dissolved. Since protoplasm developed only at a neutral reaction, its very formation had to take place in the presence of buffer compounds such as these. For this reason, we see the phosphates as a necessary part of the tissue of all forms of life and a fundamental constituent of the blood of the higher animals.

2. The union of the phosphates with fats made it possible for such phosphorylated fats to become miscible with water; and the union with sugars, to form compounds that could be absorbed through the lipoid membranes of the cell. We have, therefore, phosphorylization as an active mechanism in the absorption of fats and sugars in the intestines and the kidney tubules.

3. Phosphoric acid forms insoluble compounds with calcium and magnesium. When these chemical substances are brought together in a free condition, a precipitate is immediately formed and this deposit in the tissue may become bone, teeth, or shells. In most unicellular organisms the calcium and magnesium are held in solution in the tissue and only coagulate when the tissue is injured.

This coagulation then forms a new membrane. The same mechanism is seen in the coagulation of blood; and the calcification of limiting membrane of cells is shown by the shell of a hen's egg.

4. The phosphate ion, when joined with organic molecules increased the ease with which these molecules could combine with oxygen. The phosphates became necessary to the process by which the cells obtained their energy. Especially true is this in the case of muscle and nerve activity, when sugar, the fuel of the cell, must first combine with phosphoric acid before it can be burned.

We may recapitulate then, that the phosphate radical is fundamentally concerned in the nutrition, the metabolism, the irritability,

and the defense of the cell; and through the cell, of the organism as a whole.

Biological Implications of Metaphosphoric Acids

We now come to a consideration of the biological implications of metaphosphoric acids. Before we can consider this subject it is necessary to review the structure and chemical properties of the phosphoric acids in general, and the peculiar nature of hexametaphosphate in particular. As chemists know, the anhydride of phosphoric acid, phosphorus pentoxide, P_2O_5, combines with either one, two, or three molecules of water. With one molecule of water, two molecules of metaphosphoric acid are formed according to the following equation:

$$P_2O_5 + H_2O \rightarrow 2HPO_3$$

With two molecules of water one molecule of pyrophosphoric acid results:

$$P_2O_5 + 2H_2O \rightarrow H_4P_2O_7$$

With three molecules of water two molecules of orthophosphoric acid are obtained:

$$P_2O_5 + 3H_2O \rightarrow 2H_3PO_4$$

The orthophosphoric acid, therefore, is the completely hydrated form of the acid while the other two acids in solution tend to take up water and change to this form.

Due to the strong hydration capacities of the other two forms of phosphoric acid, the ortho- form is the one found in nature available for synthesis of organic matter. The meta- and pyro-phosphoric acids in solution undergo slow hydration to the ortho- form. This may be of fundamental importance in the biological function of these acids. It is known as reversion and hastened by an alkaline or acid reaction, and a rise in temperature. It is known that this process can be catalyzed by inorganic compounds and Kay (3) has evidence that there is an enzyme in the body able to effect this reversion.

Metaphosphoric acid, because of these strong unsatisfied hydration capacities, can combine with other molecules of itself to form polymers of complex formation, with the general formula $(HPO_3)_x$. The most interesting molecule of this group is the one where six

molecules of the simple acid are united. Commercially, this compound is obtained as the sodium salt, hexametaphosphate, first used for practical purposes by Hall (4).

Properties of Hexametaphosphate

Leaving aside the other physical properties of this salt, it is a colorless, amorphous glass, soluble to the extent of 70 per cent in water. In solution, it has two chemical properties that are responsible for its physiological action. The first is the unique quality of the compound to form a complex with calcium, magnesium, iron, and other di- and tri-valent ions, such that these metals are no longer cations but are combined with the metaphosphate radical as part of the anion. In this form these elements lose all their characteristic properties. For example, calcium in solution as sodium calcium hexametaphosphate can no longer precipitate calcium soap, calcium oxalate, or any other insoluble calcium salt.

The second property of hexametaphosphate, or more properly, of hexametaphosphoric acid, is the ability to combine with protein and, therefore, with tissue. This is shown clearly by the studies of Wilson (5) on the tanning of leather.

Physiological Action of Hexametaphosphate

The action of hexametaphosphate on living tissue arises principally from these two properties, and will be discussed under four headings: (1) the action on blood and circulation; (2) the action on the gastro-intestinal tract; (3) the action on skin and mucosa; and (4) the toxicology.

The action of hexametaphosphate on blood and circulation is first a paralysis of the heart, and then a change in the coagulability of the blood. Both phenomena are caused by the removal of calcium ions from the blood. Since the heartbeat depends on the presence of calcium ions in the blood, the heart dilates and stops when these are removed. When hexametaphosphate is injected into the blood vessels in amounts less than 20 mg. per kilo of body weight no change is noticed in the heartbeat. If an amount varying from 50 mg. to 80 mg. per kilo of body weight is injected, there is a sharp fall in blood pressure with prompt recovery. When 130 mg. per kilo are injected the result is usually fatal. The accompanying Fig. 1 shows that there is first noticed a fall in blood pressure followed by a compensatory rise, then a second fall with total cessation of heartbeat.

That this effect on the heart is due to the removal of the calcium ion by the metaphosphate is shown by the prompt recovery made by the heart when calcium is injected at the same time as the metaphosphate. If 1 mg. of calcium chloride is injected in one vein for every 1.3 mg. of hexametaphosphate injected in another vein, as much as 20 times the lethal dose of metaphosphate can be administered without any effect on the heartbeat or blood pressure.

As previously indicated blood does not coagulate when the calcium ion is removed. Blood serum normally contains 11 mg. of calcium. Of this, 6 mg. per cent is combined with blood protein, 3 mg. per cent is dialyzable but not ionized, and 2 mg. per cent is ionized. The addition of 1 mg. of hexametaphosphate per milliliter of blood

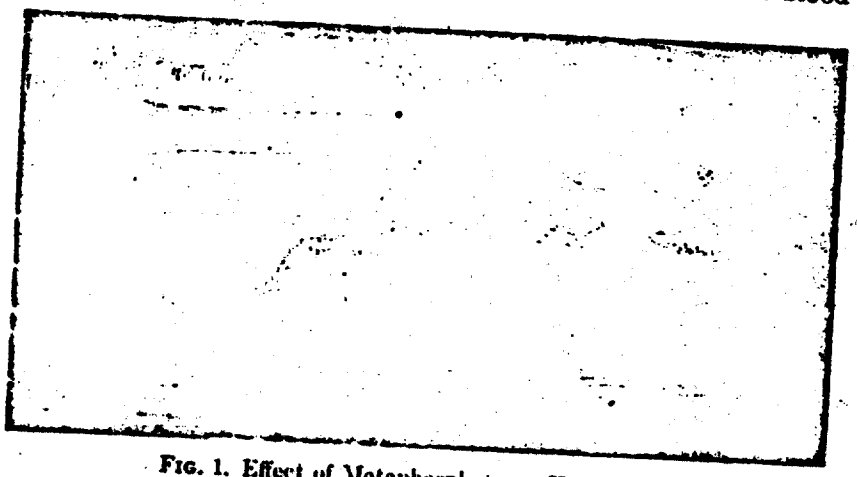


FIG. 1. Effect of Metaphosphate on Heartbeat

effectively removes the free ionized calcium, and the blood remains fluid indefinitely. Klinke (6) states that the greater part of the 3 mg. of non-ionizable calcium migrates to the anode on electrodialysis, and hence, must be in the form of an anion. This suggests the presence of a metaphosphate in the blood serum.

The action of hexametaphosphate on the gastro-intestinal tract is essentially that of the corresponding amount of the orthophosphoric acid when the amount administered is small, since the acidity of the gastric juice brings about a rapid reversion of the meta- to the ortho-acid. When larger doses are given, the hexametaphosphate acts on the calcium ion of the exposed mucosa and causes some irritation of the enteric nerves of the stomach and intestines. The

result of this is vomiting and diarrhea. Since the molecule of hexametaphosphate is large, it is very poorly absorbed, if it is absorbed at all, from the gastro-intestinal tract. In dialysis experiments with animal membranes, the amount of hexametaphosphate that passes as such, is so small that it cannot be detected. Only that phosphate, which has reverted to the ortho-radical can be found. In experiments with dogs, 50 grams have been repeatedly given by a stomach tube, the only result being prompt vomiting, no further injury ensuing. Larger amounts act in this same manner. An ounce of the commercial hexametaphosphate has been taken by man with only a pronounced laxative action being noticed.

In experiments with rats, Murray (7) fed a divided litter of twelve

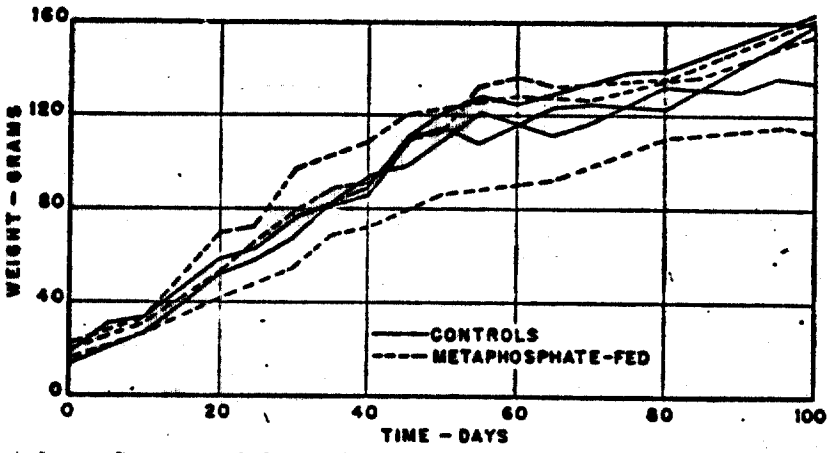


FIG. 2. Growth Curves of Rats

rats on a milk diet. To the diet of six of these he added 20 mg. of hexametaphosphate per day. The other six were kept as controls. After 100 days a pair of rats on the hexametaphosphate diet were mated. A healthy litter of 9 were born and weaned. At the end of this time the rats were autopsied. Neither gross nor microscopical examination of the liver, kidney, heart, spleen, or stomach showed injury or changes. The weight curves are shown in Fig. 2.

Five rats were fed on a diet which contained 5 per cent hexametaphosphate. On this diet the rats ingested 2 grams of hexametaphosphate per kilo of body weight per day. They remained healthy and gained in weight. When the amount of hexametaphosphate was doubled, the rats had a diarrhea for nine days, then recovered, but

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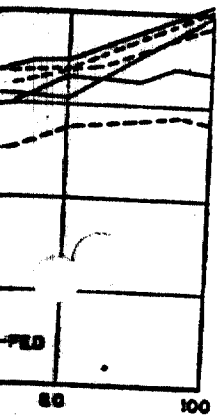

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did not gain in weight. Autopsy showed no changes, except in the lining of the intestine, which was slightly injected and hemorrhagic.

Fecal and urinary calcium were followed on these animals. The results showed a decrease in fecal calcium and an increase in urinary calcium, with a net retention of calcium. This suggests that hexametaphosphate facilitates the absorption of calcium from the intestine. A summary of these data is shown in Table 1.

TABLE 1

Calcium Metabolism of Rats on Diets With and Without Hexametaphosphate

DATE	URINARY CALCIUM (MG. DAILY)		FECAL CALCIUM (MG. DAILY)	
	Without hexametaphosphate	With hexametaphosphate	Without hexametaphosphate	With hexametaphosphate
1	0.5852	0.4280	—	—
2	0.3138	0.5825	—	—
3	0.2641	0.3400	—	—
4	0.5905	0.5280	—	—
5	0.2112	0.3748	18.5	3.03
6	0.2280	0.3465	20.3	3.03
7	0.2624	0.2477	26.4	3.04
*8	1.1165	1.7094	24.3	1.47
9	1.2480	Contaminated	25.3	3.60
10	1.2846	3.8746	19.3	2.97
11	1.7386	5.4707	14.7	2.16
12	1.8585	5.4666	16.9	2.34
13	1.7145	5.7865	17.4	2.10

Average daily calcium ingested		22.9 mg.
Average daily urinary calcium	normal	1.5 mg.
	hexametaphosphate	4.5 mg.
Average daily fecal calcium	normal	19.7 mg.
	hexametaphosphate	2.4 mg.
Average net calcium retention	normal	1.7 mg.
	hexametaphosphate	16.0 mg.

* Average calcium retention in the body computed from the last six days after diarrhea had ceased.

The absorption of the hexametaphosphate radical on four bladder-fistula dogs was studied. An increase in urine was shown but no more than an equivalent amount of orthophosphate would have given. This showed that little, if any, unreverted phosphate was absorbed. It was hoped that enough hexametaphosphate might be excreted in the urine to prevent formation of calcium stones in the ureter, but this did not occur.

Experiments by Schwartz, Jones, Mack, and Vance (8) have shown that 40 mg. of hexametaphosphate per 100 ml. of milk, can effectively prevent the coagulation of milk and the formation of curd, just as it can prevent the coagulation of the blood. This is particularly effective because the calcium is not removed from the milk and on digestion of the milk it is made available to the body. Some evidence was obtained in this work that absorption of iron was facilitated by the presence of the hexametaphosphate (see Table 2).

The main action of hexametaphosphate on the skin is again the sequestering effect of this compound on calcium ions. So fundamental is the need for the calcium ion by the body that the effect shows in many diverse and most unexpected ways whenever it is removed. The following phenomena which are affected by hexa-

TABLE 2

Analysis of Rats on Diets With and Without Hexametaphosphate

	TOTAL AVE. CALCIUM IN MG. PER GRAM OF WEIGHT	PROBABLE ERROR	AVE. TOTAL IRON IN MG. PER GRAM OF BODY WEIGHT	PROBABLE ERROR
Control	8.34	±0.196	0.058	±0.0006
Hexametaphosphate	9.20	±0.326	0.067	±0.0023
Hexametaphosphate plus iron	8.20	±0.271	0.120	±0.0024
Iron alone	8.7	±0.205	0.097	±0.0024

metaphosphate may be listed: (1) bacterial infections; (2) invisible perspiration; and (3) regeneration of epithelial tissue.

It has been pointed out previously that the calcium ion has a marked effect on the formation and stability of emulsions in the body. When this ion is in excess a water-in-oil emulsion is formed. When calcium salts are added to oil-in-water emulsions of which the living epithelial cells are a type, this emulsion changes to the water-in-oil type with the extrusion of the excess water. In other words, it is similar to the churning of butter from cream with extrusion of buttermilk. In tissue this becomes fatty degeneration and necrosis. Bacteria of the staphylococci strain can live on the normal, healthy skin without entering the deeper layers and without producing injury. Such bacteria are killed easily by sodium soap. In the presence of an insoluble precipitate of calcium soap formed in hard water, these bacteria have been shown to live for weeks with un-

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diminished vigor; therefore, they may be found in calcium soap deposits on the fibers of clothing, as determined by Gilmore (9). Such deposits of calcium soap are insoluble in water but dissolve in the oily secretions of the sebaceous glands.

We have found that when non-virulent bacteria are mixed with calcium soap and rubbed into the hair follicles, infections are obtained where they cannot be obtained by rubbing the same bacteria into a clean skin (10).

The calcium soap, therefore, produces tissue injury which permits the bacteria to grow. Hexametaphosphate, by dissolving calcium soap, prevents this entrance of bacteria into the skin and also exposes the bacteria on the skin to the destructive action of sodium soap.

Hexametaphosphate, in acid reaction, has the ability to attach itself to the protein of the skin in a more or less stable bond, depending on the pH. At a strongly acid reaction, Wilson (5) has shown that metaphosphoric acid can tan the skin. Schade and Marchionini (11) have found that normal cornified epithelium has a pH of 3.5 to 4.0. At this reaction the hexametaphosphate molecule attaches itself to the cornified layer of skin as a very thin, protective layer. It is not known whether it is due to this protective layer on the skin, or to the effect on the nerves of the skin, but experiments have shown that an application of 10 per cent hexametaphosphate will decrease the insensible perspiration by an average of 50 per cent on the resting individual at normal room temperature.

Experiments on staphylococci show that in amounts from 0.5 to 3.0 per cent hexametaphosphate agglutinates bacteria and prevents their growth but does not kill the organisms. When the cultures stand for several days and reversion to orthophosphate occurs, the bacteria again grow. The effect on isolated epithelial cells is much the same as on bacteria; but on cells attached to the body, the impermeability of the cell for the hexametaphosphate precludes any harm to the cell. Weak solutions of hexametaphosphate, therefore, are able to surround these cells with a definitely bacteriostatic and at the same time harmless environment. Placed on superficial wounds of skin or infections of mucosa, a definite prophylactic effect is obtained. Five to ten per cent solutions of hexametaphosphate are therefore of definite value in the treatment of sore throat, abrasions of the skin, first degree burns and the ordinary infections of daily life.

One point, however, in this regard needs to be considered. The injection of hexametaphosphate solutions into the skin, removes calcium from the nerve endings and throws these nerves into a state of excitation which is shown by the keen, exquisite pain experienced and by the scarletiform eruption that appears along the course of the irritated nerve. This pain makes it impossible to use the hexametaphosphate over large areas of exposed nerves where its bacteriostatic properties would be most useful otherwise.

A discussion of the toxicological properties of hexametaphosphate is rather misleading for in the usual meaning of the word, and in amounts commonly administered, none of the phosphates are toxic. Starkenstein (12) has shown that in the rabbit, the average limit to a safe dose for oral ingestion is: orthophosphate, 15 grams; pyrophosphate, 3 grams; and metaphosphate, 4 grams.

Fifteen grams of metaphosphate by stomach tube, when administered to a dog, cause vomiting, but when the same amount is given in the food it is well tolerated.

If the same ratio which obtains for the rabbit would hold for man the figures would read: orthophosphate, 450 grams; pyrophosphate, 90 grams; and metaphosphate, 120 grams.

On intravenous injection the minimal lethal dose is as follows:

		Mg. per kg.	Amount in grams per 60 kg. man
Sodium orthophosphate.....	Na_2HPO_4	4250	255
Sodium pyrophosphate.....	$Na_4P_2O_7$	70	4.2
Sodium metaphosphate.....	$(NaPO_3)_6$	130	7.80

From this, it can be seen that the toxicity of hexametaphosphate is low, for man could not tolerate 120 grams of table salt by mouth any more satisfactorily, nor could he tolerate 8 grams of table salt intravenously.

Conclusions

1. The phosphate ions are fundamentally involved in the formation of protoplasm and the origin of life.

2. The phosphates regulate neutrality of blood and tissue fluids; make the absorption of nutrients from the gastro-intestinal tract possible; regulate the oxidation of sugar in the muscle; and make possible the formation of bone and teeth.

3. The hydration capacities of the meta- and pyrophosphates suggest a mechanism that may be utilized by the body, but it is

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e 8 grams of table salt

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gastro-intestinal tract
the muscle; and make

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by the body, but it is

unknown at present. That this may occur is indicated by the ability of tissue enzymes to cause reversion of pyro- and metaphosphate.

4. Hexametaphosphate, because of its large and non-absorbable molecule, and because of the rapidity with which its reversion is brought about in the stomach, is relatively non-toxic when taken by mouth.

5. Hexametaphosphate has two actions: it combines with the calcium ion to form a non-ionized, soluble compound; and it combines with skin or mucosa to form protective films. These properties of hexametaphosphate may be used to: inhibit the heartbeat; prevent the coagulation of milk or blood; remove calcium soap deposits from hair and skin, and thereby prevent possible infection; and form protective films on skin and mucosa.

6. Hexametaphosphate is no more toxic than any physiologically active salt.

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Biochemische Z. 197: 257-8 (1928)

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Über Meta-phosphatase.

Von

Torao Kitasato.

(Aus dem Kaiser Wilhelm-Institut für Biochemie in Berlin-Dahlem).

(Eingegangen am 5. April 1928.)

Aus zahlreichen Untersuchungen, die über die Phosphatase im hiesigen Institut in den letzten Jahren ausgeführt sind, ergibt sich, daß dieses Ferment in einem großen Bereich seine Wirksamkeit entfaltet. Phosphatase greift Phosphorsäure-ester allerverschiedensten Baues an. In der aliphatischen Reihe werden von der Methyl-phosphorsäure bis zur Cetyl-phosphorsäure, also von dem niedrigst möglichen Substrat bis zu einem sehr hoch molekularen Substrat, alle geprüften Derivate hydrolysiert. Hydroaromatische Verbindungen, wie Borneol- und Cholesterin-phosphorsäure[1], werden gleichfalls angegriffen, ebenso die Phosphorsäure-ester der Phenole und hydroxylierten heterozyklischen Substanzen.

Spezifität der Phosphatase offenbart sich dagegen in stereochemischer Hinsicht[2].

Phosphatase spaltet primäre und sekundäre Phosphorsäure-ester. Besonders bemerkenswert ist, daß nicht nur Derivate der Orthophosphorsäure, sondern ebenfalls der Pyro-phosphorsäure von ihr zerlegt werden, wie dies *Neuberg* und *Wagner*[3] am Beispiel des Di-phenyl-esters der Pyro-phosphorsäure gefunden haben. Seither hat sich durch Untersuchungen von *Lohmann*[4] herausgestellt, daß auch die Pyro-phosphorsäure selbst durch ein in den Muskeln vorkommendes Ferment in Ortho-phosphorsäure übergeführt wird.

Scharf unterschieden von Ortho- und Pyro-phosphorsäure ist die *Meta-phosphorsäure*. Wegen der erheblichen Zersetzlichkeit der

[1] Nach einem neuen Verfahren gewonnen.

[2] *C. Neuberg, J. Wagner* und *K. P. Jacobsohn*, diese Zeitschr. 188, 227, 1927.

[3] *C. Neuberg* und *J. Wagner*, ebendaselbst 171, 485, 1926.

[4] *K. Lohmann*, Naturwissenschaften 16, 298, 1928.

Metaphos

meisten Meta-phosphorsäure-ester habe ich zunächst geprüft, ob rein anorganische Salze der Meta-phosphorsäure enzymatisch zu Ortho-phosphorsäure hydratisiert werden. Auf Rat von *C. Neuberg* benutzte ich für diese Zwecke das sehr beständige Natrium-hexa-meta-phosphat, $(NaPO_3)_6$, das zum Unterschied von zahlreich bekannten isomeren Modifikationen leicht löslich und beständig ist. Mit Taka-phosphatase, die vielfach zu den vorerwähnten Arbeiten verwendet worden ist, erreichte ich Umwandlung der Meta-phosphorsäure in Ortho-phosphorsäure.

Löst man in 2%igen Lösungen des Natrium-meta-phosphats Taka-phosphatase bis zu einem Gehalt von 1.5%, so wird in Gegenwart von Toluol bei 37° eine Umwandlung in Ortho-phosphorsäure erzielt, die nach 68 Stunden 20% beträgt. Während dieser Zeit bleibt die Lösung des Natrium-meta-phosphats selbst unverändert, höchstens Spuren von Ortho-phosphat sind nachweisbar. Die Überführung des Meta-phosphats in Ortho-phosphat kann leicht kolorimetrisch verfolgt werden, da reines Meta-phosphat nicht die Molybdänblau-reaktion gibt; auch Pyro-phosphat zeigt diese Reaktion nicht. Daß die enzymatische Umwandlung des Meta-phosphats in Ortho-phosphat direkt und kaum über die Stufe des Pyro-phosphats geschieht, darf wohl daraus geschlossen werden, daß zu keiner Zeit in den Lösungen mit Zinksulfat ein gegen verdünnte Essigsäure resistenter Niederschlag erzeugt werden kann.

Versuche mit tierischen Organen (Leber und Niere des Kaninchens) sowie mit Hefe haben gleichfalls die enzymatische Umwandlung des Meta-phosphats zu Ortho-phosphat ergeben; die Wirkung scheint aber schwächer als bei der Taka-phosphatase zu sein.

Mit der eingehenden Untersuchung dieser Phosphatase-wirkung bin ich beschäftigt.

Compt. Rend. Soc. Biol. 108 : 678-80 (1931)

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Société de biologie de Lettonie.

Séance du 5 décembre 1930.

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Présidence de M. A. Netter. 517

LES VARIATIONS DU TAUX DU PHOSPHORE SANGUIN SOUS L'INFLUENCE DE L'ABSORPTION D'ACIDE PHOSPHORIQUE CHEZ L'HOMME,

par MARCEL LABBÉ, M. FABRYKANT et C. ZAMFIR.

L'influence exercée par l'absorption *per os* des phosphates sur le taux du phosphore minéral sanguin a fait l'objet d'études de plusieurs chercheurs qui le plus souvent ont constaté dans ces conditions une augmentation de la phosphatémie. Certains d'entre eux ont même trouvé que l'ampleur de cette réaction phosphatémique se montre constante chez un sujet donné.

Nous avons soumis à l'expérience 3 sujets normaux et 5 diabétiques. Le phosphore était administré non pas sous forme de phosphates mono- et di-sodique, comme cela a été pratiqué par différents auteurs, mais sous forme d'acide phosphorique officinal. L'introduction dans l'organisme du phosphore sous cette forme permet d'éviter les diarrhées possibles après l'ingestion des phosphates et la résorption du phosphore se fait ainsi dans des conditions normales. De plus, l'administration d'acide phos-

phorique nous a offert l'occasion d'examiner simultanément avec le phosphore sanguin les variations de la réserve alcaline. Cela représente un intérêt spécial, vu que certains auteurs admettent que les modifications de la phosphatémie résultent de l'état de l'équilibre acido-basique du sang. Pour les déterminations du phosphore sanguin nous avons employé la méthode de M. Javillier et M. Fabrykant. Les prélèvements de sang ont été faits chez tous les sujets le matin, à jeun depuis 12 heures et après 1 heure de repos. Le deuxième prélèvement a été pratiqué 1 heure après l'absorption de l'acide phosphorique. Les résultats de nos expériences sont réunis dans le tableau ci-joint. Les chiffres de P sont indiqués en mgr. par litre de sang total ; la réserve alcaline en volumes de CO^2 pour 100 c.c. de plasma.

Nos	P total	P minéral	Réserve alcaline
1 a	383	32,31	65,3
b	385	46,67	64
2 a	373	38,18	65,2
b	370	44,21	65,7
3 a	354	33,60	64,2
b	353	38,18	64,4
4 a	373	40,97	50,5
b	373	36,52	52
5 a	400	30	42
b	400	31,69	
6 a	390	34,29	57,4
b	392	37,33	55,5
7 a	353	35	58
b	350	36,52	60
8 a	364	31,94	37,1
b	380	45,35	52,5

a : à jeun ; b : 1 heure après absorption de 60 gouttes d'acide phosphorique officinal dans 200 c.c. d'eau.

Chez nos 3 sujets normaux nous avons trouvé 1 heure après l'absorption de 60 gouttes d'acide phosphorique officinal dans 200 c.c. d'eau, une augmentation du phosphore minéral variant de 4,58 mgr. à 14,36 mgr., soit de 13,63 à 44,44 p. 100 de la valeur initiale. Par contre, les variations du taux du phosphore total se sont montrées insignifiantes et sans proportionnalité avec celles du phosphore minéral.

Chez les diabétiques (cas 4 à 7), nous avons constaté des réactions irrégulières : l'abaissement du phosphore minéral dans certains cas, dans d'autres au contraire, une légère augmentation de la phosphatémie. Par ailleurs, chez les diabétiques tout comme chez les sujets normaux, le phosphore total s'est trouvé pratiquement inchangé. Dans le dernier cas de notre statistique, le

sujet a ingéré une dose plus forte d'acide phosphorique (80 gouttes) ; celle-ci a entraîné une élévation, non seulement du phosphore minéral, mais aussi du phosphore total.

En résumé, l'absorption de 60 gouttes d'acide phosphorique par un sujet normal, à jeun depuis 12 heures, détermine, en l'espace d'une heure, une élévation du phosphore minéral du sang, dont l'ampleur varie d'un sujet à l'autre. Chez les diabétiques les réactions sont moins nettes dans les cas positifs, mais d'une façon générale irrégulières. L'absorption d'une dose plus forte d'acide phosphorique (80 gouttes) peut entraîner également une élévation du phosphore total.

Quant à la réserve alcaline, chez le sujet normal de même que chez le diabétique, nous n'avons pu mettre en évidence aucun rapport entre ses variations et celles de la phosphatémie (1).

Sur un développement atypique du corps mitochondrial chez le mâle (Faux-bourdon) de l'Abeille domestique (*Apis mellifica*, Hyménoptères),

par Janina Orska.

Pendant mes recherches sur la spermatogenèse du mâle de l'Abeille j'ai observé un développement du corps mitochondrial dans les spermatides de cet Insecte, qui diffère du développement du même corps chez beaucoup d'autres Insectes (Coléoptères, Hémiptères, Lépidoptères, etc.). Ordinairement il y a dans les jeunes spermatides un corps mitochondrial impair, qui se divise plus tard en deux parties égales et symétriques avec une structure intérieure spécifique. Ces deux parties s'allongent de plus en plus, en même temps et également, et forment enfin la queue du spermatozoïde, qui a, chez les Insectes, une longueur considérable.

Chez les mâles de l'Abeille, les mitochondries forment, après la dernière division des spermatocytes, un corps mitochondrial impair, qui se divise dans les spermatides plus âgées (fig. 1) en deux parties égales (*pm*, *sm*), comme chez les autres Insectes. Ces deux parties s'allongent en même temps dans une spermatide plus âgée (fig. 2) et prennent la forme de deux fuseaux de longueur égale. A partir de ce moment le comportement de chacune de ces deux parties du corps mitochondrial est différent, phénomène inconnu dans la spermatogenèse des autres Insectes.

(1) Les déterminations du phosphore ont été faites par l'un de nous au laboratoire du Pr M. Javillier.

Proc. Soc. Exp. Biol. Med. 44 (2): 554-5 (1940)

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Use of Sodium Hexametaphosphate as an Anticoagulant.

CLARENCE E. LARSON.

From the Chemistry Department, College of the Pacific.

Sodium hexametaphosphate has recently become established as an effective agent in reducing calcium ion concentration. Most of the research work on the compound has been done in the field of industrial chemistry where it has demonstrated its use as an outstanding water softener.

In this report we are concerned with the mechanism by which this compound reduces calcium ion concentration. The consensus seems to favor the formula $(NaPO_3)_6$ or $Na_6P_6O_{18}$. This compound can react with calcium ion to form $Na_2Ca_2P_6O_{18}$ thus leaving the calcium in the form of a complex since the compound ionizes into 2 sodium ions and the negatively charged $Ca_2P_6O_{18}$ ion. The dissociation of this complex ion to yield calcium ion is so slight that the addition of sodium hexametaphosphate depresses the calcium ion to such an extent that it will dissolve the most insoluble calcium salts such as the carbonate, the oxalate, and the phosphate. At present it is impossible to express mathematically the equilibrium constant for the dissociation of the complex ion but indications are that its effective value approaches 10^{-15}.

Comparatively little work has been done on the biochemical and physiological properties of the compound. This paper represents the first report of results of work in progress in our laboratories on the application of properties of sodium hexametaphosphate to the fields of biology and medicine.

The effect of this compound on the clotting of blood was tested by the following method. A stock solution of 20% sodium hexametaphosphate was made up. Varying quantities were added to calibrated tubes so that the final concentrations after blood was added ranged from 0.1 g per 100 cc to 2.0 g per 100 cc. Blood was allowed to flow directly from the sheep being used into the tubes. Each tube was rocked carefully to insure complete mixing. A control tube containing no reagent was treated in the same way. The control tube showed clotting in 5 minutes. All tubes containing sodium hexametaphosphate remained unclotted. Experiments were also performed on human blood with the same results. Concentrations of sodium hexametaphosphate less than 0.1 g per 100 cc slowed the process of clotting but did not prevent it entirely as was the case of the higher concentrations.

It is well known that the use of excess quantities of an anticoagulant such as sodium oxalate interferes with certain analytical procedures such as deproteinization. Folin-Wu filtrates were made on the samples but no interference with successful deproteinization was detected at any of the concentrations used.

These experiments indicate that sodium hexametaphosphate can be added to the list of blood anticoagulants, and may have certain advantages over existing agents.

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quantity to be detected by their method of flame excitation of the spectrum. Furthermore they say that Ba is not present in the chorioids of human, sheep, pigs, horses, dogs and many sea fish. Ramage and Sheldon failed to find this element in the retina although they could detect it in the iris and the pigment of the chorioid. It is of some significance that they found Ba in the chorioids of all neat cattle beyond 3 years in age. Gerlach and Müller[2] examined eyes from a wide variety of animals including man and discovered Ba in the chorioid of most of them. It was not present uniformly in human chorioids and there appear to be no age peculiarities in its distribution. These writers also describe Ba in the retinæ of ostrichs, rabbits, cats, cattle and in one human.

The material in the present series consisted of 19 pigs, 17 ox, 24 sheep and 12 kitten eyes. The spectrographic method used was that described by us in an earlier paper (Scott and Canaga[3]). We used as identifying lines the 4535.5 and 4934.1 Å. These lines are quite sensitive and can be definitely located with little trouble. An

* Aided by a grant from the Josiah Macy, Jr., Foundation.

1 Ramage and Sheldon, *Nature*, 1931, 128, 376.

2 Gerlach and Müller, *Arch. f. path. Anat. u. Physiol.*, 1936, 296, 588.

3 Scott and Canaga, PROC. SOC. EXP. BIOL. AND MED., 1939, 40, 275.

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J. Pathol. Bact. 52: 17-24 (1941)

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EXPERIMENTAL PHOSPHATE NEPHRITIS IN THE RAT

561

DAVID MCFARLANE

From the Department of Pathology, University of Glasgow and Western Infirmary

(PLATES IV-VI)

THE purpose of this paper is to describe and discuss the nature, localisation and causation of the earliest changes, regarded as significant, produced in the kidney of the rat by the administration of phosphate. This subject has been most fully described by Mackay and Oliver (1935) and by Duguid (1936). These authors, using oral and intraperitoneal administration respectively, have both obtained renal lesions, but they differ in opinion as to the exact nature of these changes. In the following experiments both methods of administration were employed; the early changes obtained in both were the same.

In order to clarify the later discussion it is necessary to give some account of the distribution of the nephrons in the rat kidney (figs. 1 and 2). As in all mammals, the first tubule on leaving the glomerulus pursues a closely convoluted course, then, without alteration in the character of the cellular lining, changes to the straight part which passes vertically in the direction of the medullary pyramid (Peter, 1909; Huber, 1932; Pai, 1934-35). In the majority of nephrons these segments change suddenly at approximately one level to the narrow descending limb of Henle. In a large proportion the narrow limb is comparatively short (type 2) and changes again sharply to the broad limb of Henle, which immediately bends round as the "loop" and ascends again towards the cortex, reaching the neighbourhood of the glomerulus whence the tubule started; there it is attached to the efferent arteriole before passing on as the second convoluted tubule. The narrow limbs of type 1 nephrons are much longer and descend for variable distances towards the medullary papilla, then turn at the "loop", still as narrow limbs, and ascend towards the cortex, changing suddenly to broad limbs about the same level as the loops of type 2. In a certain proportion of nephrons (type 3), unascertained in the

Phos

EXPERIMENTAL PHOSPHATE NEPHRITIS

FIG. 1

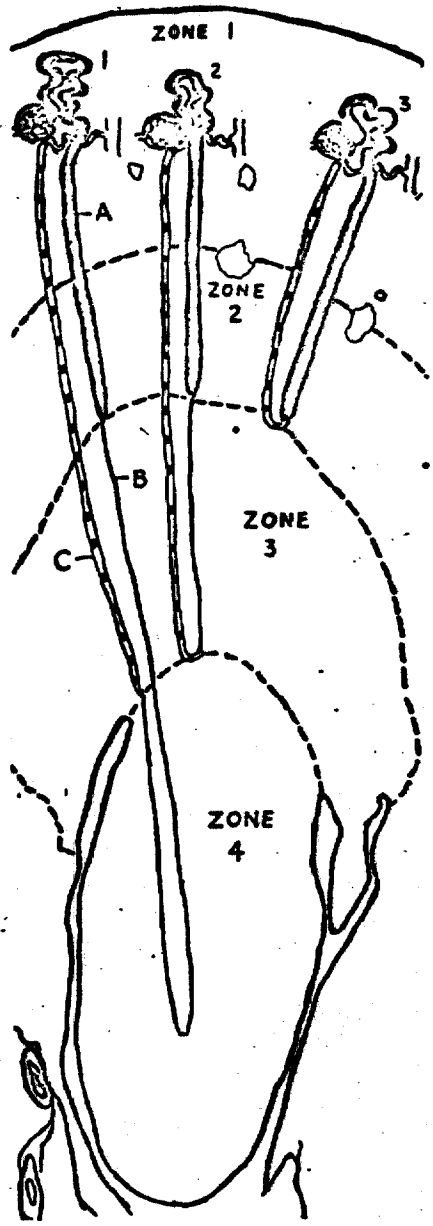

FIG. 2

FIG. 1.—Transverse hemisection of normal rat kidney. The four zones described in the text are clearly distinguishable. ×12.

FIG. 2.—This shows the distribution of the various parts of each of the three types of nephrons (1, 2 and 3) in the different zones of the kidney. A = straight part of 1st convoluted tubule; B = narrow limb of Henle; C = broad limb of Henle and 2nd convoluted tubule. ×12. Nephrons much more highly magnified.

Zone 1 = outer cortical zone (cortex—Peter)
Zone 2 = inner cortical zone (outer stripe of outer zone of medulla—Peter)
Zone 3 = outer medullary zone (inner stripe of outer zone of medulla—Peter)
Zone 4 = inner medullary zone (inner medullary zone—Peter)

rat, there is no narrow limb, the straight part of the first tubule changing directly into the broad ascending limb within the cortex about the same level as the change from the descending straight first convoluted tubule to the narrow limb of Henle in types 1 and 2.

If serial sections cut transversely to the axis of the medullary pyramid from the cortex downwards are studied, four zones can be distinguished as a result of the above arrangement of the nephrons.

Zone 1. In the outer cortex the arrangement is labyrinthine, the predominant tubule being the first. This zone is 2-3 mm. in depth and merges insensibly with **zone 2**, in which, especially towards the medulla, the majority of the tubules are cut transversely and are either descending straight parts of first tubules, ascending broad limbs or collecting tubules. The descending straight parts of first tubules form the predominant histological feature. This zone is 1·0 to 1·5 mm. in depth. **Zone 3.** The histologically characteristic descending first tubule by a sudden change has disappeared, being replaced by the narrow limbs of Henle. The other tubules seen in this zone are the collecting tubules and the broad limbs of Henle; the latter are numerous, fairly large and histologically characteristic, and form the predominant tubule in this zone. This zone is 2·0 to 2·5 mm. in depth. **Zone 4.** By another sudden change all broad limbs have disappeared and only narrow limbs are seen, along with collecting tubules which diminish in number and increase in size as the apex of the pyramid is approached. This zone is 3·5 to 5·0 mm. in depth.

On naked eye examination of the kidney, zones 3 and 4 are easily identifiable; the latter as a pale pinkish grey triangle with the medullary papilla as its apex, the former as a slightly darker brownish zone arching over the base of the other. This in turn is arched round by the well demarcated darker cortex. There is no sharply defined boundary between microscopical zones 1 and 2. Mackay and Oliver follow Peter in describing zone 2 as the outermost part of the medulla; but as this zone contains as its most characteristic component the terminal portion of the typical cortical (*i.e.* the first convoluted) tubule, it seems more reasonable to call it the inner cortical zone. In larger rodents like the rabbit, as in man, these tubules do not form a continuous zone but are present only in the medullary rays. The above micro-anatomical findings are summarised in the accompanying table, in which the nomenclature used by Peter is included for convenience.

The most important fact emerging from the above considerations is that by studying serial sections it is relatively easy to determine the exact localisation of morbid changes in the various segments of the nephron.

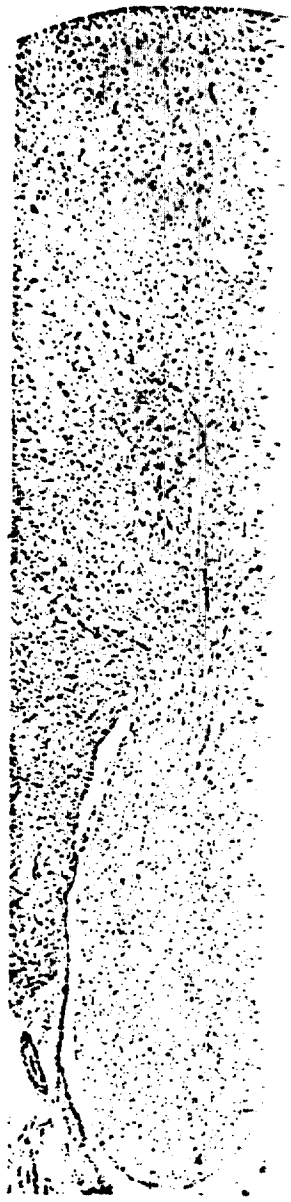

FIG. 1

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Zone 2 = inner co
Zone 3 = outer m
Zone 4 = inner m

TABLE

Zonal nomenclature and distribution of nephrons in the rat kidney

Nomenclature of Peter			Distribution of nephrons in the four zones above-described	Nomenclature adopted for the zones of the rat kidney	
CORTEX			**Zone 1** *Labyrinthine 1st tubules* 2nd convoluted tubules Broad limbs of Henle Collecting tubules	OUTER ZONE	CORTEX
MEDULLA	OUTER ZONE	OUTER STRIPE	**Zone 2** *Descending straight limbs of 1st tubule* Broad limbs of Henle Collecting tubules	INNER ZONE	
		INNER STRIPE	**Zone 3** *Broad limbs of Henle* Narrow limbs of Henle Collecting tubules	OUTER ZONE	MEDULLA
	INNER ZONE		**Zone 4** Collecting tubules Narrow limbs of Henle	INNER ZONE	

In column 4 the italicised segment of the nephron is the characteristic component of each of the first three zones.

Experimental

The type of rat used was the albino *Mus norvegicus*, a pure-bred Wistar strain.

The first few kidneys were fixed in Zenker's solution but this did not prove satisfactory, contrary to previous experience. The remainder of the kidneys were fixed in corrosive-formol (saturated aqueous corrosive sublimate 90 c.c., formaldehyde (40 per cent.) 10 c.c. *plus* 5 per cent. of acetic acid) for 8 hours and then transferred to a saturated aqueous solution of corrosive sublimate for 48 hours (Lendrum, 1941).

Group 1. *Intraperitoneal administration.* Eight rats were used. These received from 0·1 to 0·36 g. per 100 g. body weight of NaH_2PO_4 in 20 per cent. solution. The rats did not tolerate this treatment very well and showed marked signs of air hunger and general uneasiness for varying periods after dosage. They were killed at periods from 12 hours to 3 days: 5 of them showed slight but definite early changes.

Group 2. *Oral administration.* Fifteen rats were used. They were kept in individual cages and received a measured volume of milk containing 3·3 per cent. of NaH_2PO_4. The daily intake of phosphate was thus easily calculated and lay approximately within the limits of 0·34 and 1·2 g. per 100 g. body weight per day. The rats took the milk mixture readily and showed no untoward symptoms. They were killed at periods ranging from 7 hours to 42 days. Two of the rats of this group showed no lesion; one of these was killed in 7 hours and the other had the lowest daily intake of phosphate over the 7-day period of the experiment.

The early lesions of phosphate nephritis

Macroscopically, the earliest lesion (12 hours) is congestion of the outer medullary zone. This is followed, after 17 hours, by the appearance of minute white flecks in the outer medullary zone near its junction with the inner medullary zone. These increase in number and size and after 2 days become partially confluent, forming a narrow white line which consists of thin white streaks radiating towards the capsule of the kidney (fig. 3). Further increase in size and confluence of the white areas results (4-7 days) in progressive alteration of the entire medullary zone into a broad white band (fig. 4). No other naked eye abnormality is present.

Microscopically, the first changes considered to be significant (12-17 hours) are nuclear pyknosis, coagulative necrosis and early breakdown of the cells of the broad limb of Henle (fig. 5). As ascertained by serial sections, these are localised to a segment of the broad ascending limb 200-400 μ in length, commencing about 100-200 μ from the junction of inner and outer medullary zones. The remainder of the broad limb of Henle beyond the segment of damage is unchanged. At this stage there is no cellular infiltration around the damaged tubules. The first convoluted tubules show some swelling of the cells and detritus is present in the lumina, but there is no dilatation, pyknosis or necrosis. The narrow limbs of Henle contain many casts, mainly hyaline, some granular.

At 2 days more complete degeneration of the tubular epithelium is present, including disappearance of nuclei and the formation of granular casts from the necrotic cells (figs. 7 and 8). The casts appear to occlude, partially or totally, the lumen of the damaged tubules and they can be seen in all stages of calcification. Infiltration by small round and larger endothelioid cells is now noted around the damaged tubules. At this stage other features are seen in the cortex and inner medulla. In the cortex a number of the descending straight limbs of the first convoluted tubules show marked dilatation, their brush border being very distinct; a few of the broad limbs of Henle in this zone also show marked dilatation. Early necrotic changes are evident in some of these dilated tubules, many of which are descending straight limbs of first convoluted tubules, although some may well be broad limbs of Henle. Subsequent stages (4-7 days) are characterised by aberrant hyperplastic changes, both in the surviving epithelium and in the interstitial tissue of the specific segment of maximal damage (figs. 10 and 11). Many multinucleate giant cells make their appearance and calcification is evident, although its intensity varies from case to case. Dilatation of both the descending straight limb of the first convoluted tubule and the narrow limb of Henle is very marked.

EXPERIMENTAL PHOSPHATE NEPHRITIS

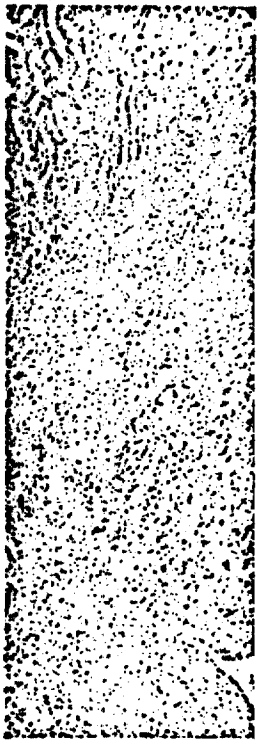

FIG. 6.

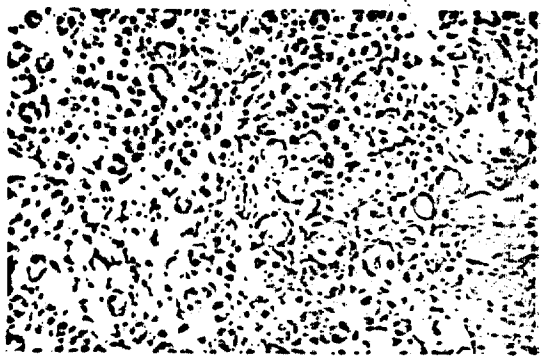

FIG. 7.

FIG. 8.

FIG. 9.

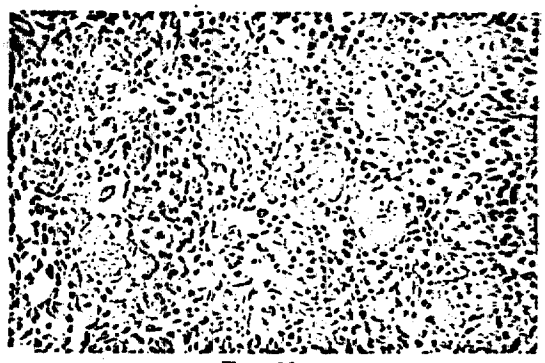

FIG. 10.

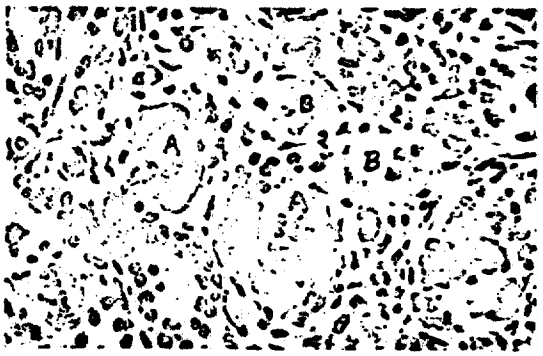

FIG. 11.

Plate VI

Fig. 6.—2-day lesion. Vertical section. At the top of the field the dilated terminal portions of undamaged 1st tubules are seen (zone 2). The change from the inner cortical zone (zone 2) to the outer medullary zone (zone 3) is well demarcated. The cortical half of the latter zone is free from damage, while the medullary half shows well marked lesions. These cease abruptly and the inner medullary zone (zone 4) is undamaged. ×27.

Fig. 7.—2-day lesion. Transverse section through the level of maximal incidence of the lesion in the outer medullary zone (zone 3). Many normal tubules (small, circular and well defined) are present. In the centre of the field there are several necrotic tubules (large and irregular) around which there is a distinct cellular infiltration. ×135.

Fig. 8.—2-day lesion. Higher power of part of fig. 7. This shows the normal tubules (A) and damaged tubules (B), the latter in various stages of coagulative necrosis. Peritubular cellular infiltration is present. ×270.

Fig. 9.—4-day lesion. Vertical section. This shows involvement of almost the complete outer medullary zone (zone 3) in the destructive process. The inner cortical zone (zone 2) at the top of the field and the inner medullary zone (zone 4) at the bottom of the field are clearly not involved. ×27.

Fig. 10.—4-day lesion. Transverse section of the zone of maximal incidence of the lesion in the outer medullary zone (zone 3). Massive cellular infiltration, with hyperplasia and calcium deposition, leads to complete distortion of the zone (*cf.* fig. 7: the sections are taken at the same level and at the same magnification). ×135.

Fig. 11.—4-day lesion. Higher power view of fig. 10, showing giant cells (A), necrotic tubules (B) and infiltration of the area by large cells of endothelioid nature. ×270.

EXPERIMENTAL PHOSPHATE NEPHRITIS

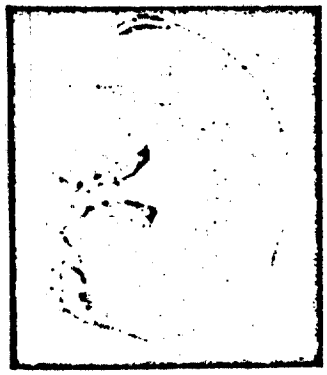

FIG. 3.—2-day lesion. Longitudinal hemisection. The lesion is clearly seen partially encircling the pale triangle of the medullary pyramid. ×2.

FIG. 4.—4-day lesion. Longitudinal hemisection. The lesion is situated in the same region as in fig. 3 but is of considerably greater intensity. ×2.

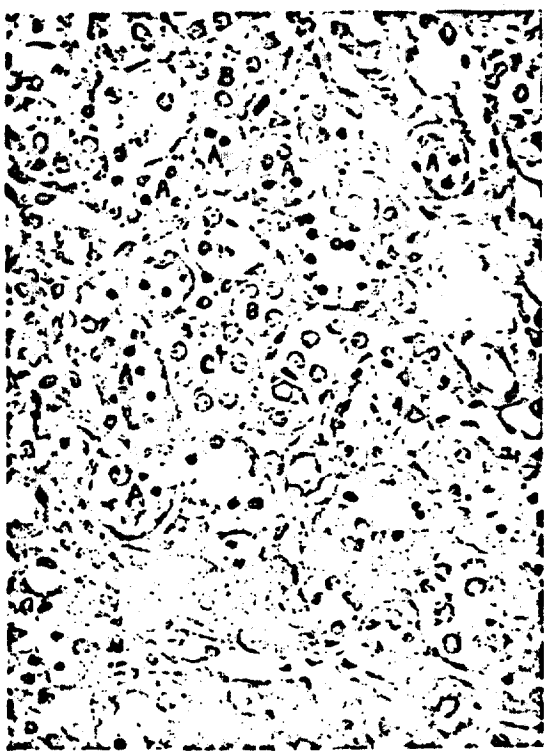

FIG. 5.—Transverse section of the outer medullary zone (zone 3) 200 μ from its junction with the inner medullary zone (zone 4), showing pyknosis of the majority of the nuclei of the cells of the broad limb of Henle (A). Undamaged broad limbs of Henle (B) and collecting tubules (C) are also seen. ×300.

Discussion

Four points arising from the present experiments require consideration.

1. The localisation of the primary lesion in phosphate nephritis to the broad limbs of Henle places it in a different category from most comparable experimental nephropathies, since in practically all of these the primary damage is restricted to the first convoluted tubule.

2. The results demand an interpretation of histogenesis differing from that of Duguid.

3. The primary lesion is definitely in a different zone and in a different segment of the nephron from that described by Mackay and Oliver.

4. The specific localisation of the early lesion and the fact that it is dependent on excess of phosphate indicate that the mechanism involved in the production of the necrosis may well be associated with the acidification of the glomerular filtrate in the damaged segment.

Duguid, using 38 rats, obtained lesions in only 6, 3 of which showed necrosis of tubular epithelium, the changes described affecting the first convoluted tubules diffusely without any tendency to localisation. The histogenesis as put forward in his paper was a primary breakdown of the luminal border of the cells in the first convoluted tubules, the debris so produced passing along the lumen of the tubule and becoming impacted in the terminal portion of the first convoluted tubules and in the narrow limbs of Henle. Duguid presumes that this debris, which has the appearance of being packed in the tubules at the cortico-medullary junction, and the debris that shows calcium deposition in his more prolonged oral administration experiments (Duguid, 1933-34) have the same origin. This presumption is invalidated by two considerations. Firstly there is an absence of experimental data covering the all-important period between the acute early lesion and the more advanced lesion with calcification, and secondly complete and different findings are presented.

In the present experiments, after phosphate administration, rats of groups 1 and 2 showed very early lesions which were similar in every respect. These involved selectively a specific proximal segment of the broad ascending limb of Henle, commencing with pyknosis and progressing to complete loss of nuclei and breakdown of cells. All stages from these early lesions to the more advanced lesions with calcification were obtained in group 2 rats by oral administration. By correlation with earlier lesions, it was clearly seen, in either serial or vertical sections, that the calcium was being deposited in the necrotic cells of the specifically susceptible

segment of the broad limb of Henle. The appearance of plugging of the narrow limbs of Henle by casts (which incidentally never showed calcification) is probably an artefact, possibly due to fixation of albuminous fluid.

Mackay and Oliver, whose description is full, describe the development of the lesion as follows. Primary necrosis of the terminal portion of the descending straight limb of the first convoluted tubule was followed rapidly by hyperplasia of peritubular elements and cellular infiltration; massive aberrant hyperplasia of surviving tubular epithelial cells then took place. Giant cells appeared and calcium was deposited in the necrotic debris, with great resulting distortion of this zone. Using the nomenclature of Peter, already outlined, Mackay and Oliver describe the lesion as being confined to the outer stripe of the outer zone of the medulla, about which they say (p. 327): "In a section of the normal rat kidney this is that clearly delimited band of tissue, lying immediately beneath the cortex, which contains as its characteristic component the terminal divisions of the proximal convoluted tubule". In criticism of this, it is to be noted that the zone of the descending straight limbs of the first convoluted tubules is not clearly "delimited" either macro- or microscopically. It should be mentioned that in the present experiments one kidney (42 days) shows a definite naked eye and microscopical lesion of the inner cortical zone as well as a marked lesion of the outer medullary zone, but as no similar cortical lesion occurs in any of the earlier experiments and as it is much smaller than the lesion of the outer medullary zone, it must be considered a late change.

Comparison of the findings of the American workers with those of Duguid and myself shows the production of similar types of lesions, but Mackay and Oliver state definitely—although their illustrations do not help in this respect—that the changes are localised to the terminal divisions of the proximal convoluted tubule. Duguid (1935), on the other hand, although he does not specifically discuss the localisation of the lesion to any one zone of the kidney, publishes an excellent photograph of an 18-day lesion which shows localisation of the more chronic lesion to the outer medullary zone. This accords very well with the findings at similar stages in the present work.

There are two possibilities which would explain the above anomalous position. Mackay and Oliver have either wrongly localised the lesion or they have actually obtained it in the site they mention; although, after having used experimental technique similar to that used in the present work, it seems highly improbable that they would obtain lesions similar to those of Duguid and myself which involved a different segment of the nephron.

So far there has been no attempt to account for this very specifically localised necrotising process, although by using various salts of H_3PO_4 Mackay and Oliver have shown beyond doubt that it is due to the PO_4 and not to the basic radicle. In the body fluids the salts of H_3PO_4 are mainly B_2HPO_4 (generally Na_2HPO_4); this salt closely approximates to the *p*H of the tissue fluids. The B_2HPO_4 of the glomerular filtrate is changed somewhere in the nephron proximal to the collecting tubules to BH_2PO_4; this change is not only one of the major mechanisms for the conservation of base by the body, but is also largely responsible for the acidification of the urine. Under normal conditions this conservation of base and resulting acidification have no damaging effect on the tubular epithelium of the nephron, but it is possible that if there be a sufficient increase in phosphate intake the glomerular filtrate could contain sufficient phosphate which, when concentrated and changed to BH_2PO_4, would so alter the physical properties and *p*H of the filtrate that necrosis of epithelial cells would supervene.

The alteration of the physical properties by concentration is almost certainly incapable, *per se*, of causing specifically localised necrosis, as Walker and Hudson (1937), using frogs and necturi, have shown that the concentration of the glomerular filtrate varies at the same level from nephron to nephron. The mechanism of this variation may be deduced from the work of Richards who, by direct observation, has shown that only a small number of the total glomeruli in the frog's kidney are functioning at one and the same time and that each of these functioning glomeruli has a different blood flow. This latter fact means that there is a different volume of filtrate escaping into each tubule. The amount of fluid filtering into a tubule determines the rapidity with which it passes along that tubule. A large volume of rapidly flowing filtrate offers to the tubular epithelium a smaller surface area of filtrate in proportion to volume for less time than does a smaller volume of more slowly flowing filtrate, and would obviously suffer less concentration.

In mammals the site of acidification of the glomerular filtrate within the nephron has not so far been definitely localised, although Dunn and Polson (1926), in a paper describing the only other nephropathy in which the damage is clearly and almost wholly restricted to the broad limb of Henle, have suggested that it may be acidification of the glomerular filtrate which leads to the precipitation of urate and so to the production of the lesion in this segment in experimental uric acid nephritis.

Montgomery and Pierce (1937) have shown that in amphibia (frogs and necturi) acidification takes place in a specific segment of the distal tubule 1/5th the length of, and situated in the distal half of the distal tubule.

If acidification of the urine in mammals were the function of the broad limb of Henle, this would offer a more likely mechanism for such a specifically localised lesion as occurs in phosphate nephritis. The factors which suggest that such functional specificity may well exist are summarised as follows.

1. It is known that in certain amphibia acidification is confined to a specific segment of the distal tubule. It is probable that in the more highly specialised mammals such a localisation also exists.

2. Phosphates form an integral part of the mechanism for the acidification of the urine.

3. In experimental phosphate nephritis a specifically localised lesion is produced.

The following tentative conclusion is drawn from these considerations. The site of acidification of the glomerular filtrate in the nephron of the rat kidney is that segment which is the site of necrosis in experimental phosphate nephritis, namely a proximal segment of the broad limb of Henle 200-400 μ in length situated 100-200 μ from the junction of the inner and outer medullary zones.

Summary

The early lesions produced in the rat kidney by intraperitoneal injection and by oral administration of NaH_2PO_4 are similar. They are restricted to a specific segment of the broad limb of Henle in the outer medullary zone. This segment is 200-400 μ in length and is situated 100-200 μ from the junction of the inner and outer medullary zones. It is suggested that it is this segment which is the site of acidification of the urine in the rat.

I wish to acknowledge my indebtedness to Professor Shaw Dunn, at whose suggestion this investigation was undertaken, for his guidance and help throughout the work. To the Rankin Medical Research Fund thanks are accorded for financial assistance.

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ON PHOSPHATES AND PHOSPHORIC ACID AS DIETARY FACTORS IN THE CALCIUM BALANCE OF MAN

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(Received for publication August 6, 1952)

It is well known that the skeletal calcium can be drawn upon in the acid-base regulation of the organism. In the days of severe diabetic ketosis extreme values for calcium in the urine were frequently observed. Early experiments with mineral acids also indicate that the skeletal calcium can be used for neutralisation of excess acid. Ammonium chloride has been used for the study of experimental acidosis, and a heavy load of ammonium chloride is followed by an increase in the urinary calcium already on the first day.

The most instructive experiments in this direction are those reported by Dennig et al. (1929), Farquharson et al. (1931) and Albright & Reifenstein (1948).

Phosphoric acid and phosphates as introduced by variations in the composition of diets can be thought to influence Ca-metabolism in two ways. Phosphoric acid in excess might carry calcium with it in the urine for neutralisation purposes. Phosphates which readily form insoluble Ca salts above a certain pH might reduce the absorption of calcium.

A number of earlier studies have given varying results. Farquharson et al. (1931), in their well known studies of calcium metabolism on low Ca diets, observed no effect of high doses of neutral or acid phosphates on the fecal or urinary calcium. In some experiments with varying protein level in the diet, an increase of the daily intake from 40 up to 100 g protein daily was followed by a considerable increase of urinary calcium.

McCance, Widdowson & Lehmann (1942) observed a slight increase in Ca absorption and an even slighter increase in urinary Ca following the transition of their subjects from a moderate to a very high protein diet.

Mellanby (1949) concludes that phosphates added to the diet depress the absorption of calcium. This contention is based on experiments in vitamin-D deficient puppies. However, the experimental evidence to support such a conclusion is weak. On the contrary, the material presented in his publication rather points to no effect of phosphate in these experiments. Baylor et al. (1950) gave 2015 mg P as acid phosphate by mouth daily for 24 days to a 57 year old osteoporotic female. No significant increase was observed in fecal calcium during phosphate administration, but marked reduction of urinary calcium occurred. The decrease in urinary calcium as compared with control periods was seen also when 534 mg Ca as gluconate was injected intravenously daily for 8 days during phosphate period. Significant decrease in urinary calcium was observed in children

Phos

following addition of sodium glycerophosphate to the low-calcium diet. Knapp (1947).

Recently Leichsenring et al. (1951) published experiments in which they observed that the addition of 600 mg P as dibasic phosphate reduced the calcium absorption by about 200 mg daily. They feel that variations in the phosphate content of the diet may be a factor of considerable importance in calcium metabolism.

These, as well as most other studies in man, were of short duration.

For some years, protracted Ca-studies have been carried on in this laboratory. The purpose has inter al. been to establish a better basis for the evaluation of the calcium requirement of man. The experiments will be reported in full later.

In the course of these investigations, which up to now have been conducted on 30 men in all (continuous observations for more than two years in some of them), many types of individual variations have been observed.

In the light of the recent publications on the effect of phosphates on the calcium balance, some studies were included in our investigations. Since the results of our studies indicate clearly that variations in the intake of phosphate or phosphoric acid, as can occur in various dietaries, have no influence on the calcium balance, a publication of these results seems warranted at this time.

EXPERIMENTAL

The experiments to be presented are the following.

1. Brief ingestion (one week) of 2.37 g orthophosphoric acid daily (750 g P) in four adult men (28, 29, 32 and 43 years of age).
2. Protracted ingestion (four weeks) of 3.15 g of orthophosphoric acid (1000 mg P) daily in two adult men (40 and 43 years of age).
3. Protracted ingestion (eight weeks) of 0.8 g of orthophosphoric acid (250 mg P) daily in four adult men (20, 28, 46 and 56 years of age).
4. Protracted ingestion (twelve weeks) of a neutral (Na -K) phosphate mixture (600 mg P) daily in two adult men (20 and 43 years of age).

All these experiments were carried out in experimental persons who had been observed with regard to their calcium balance for months and years prior to the experiments with addition of phosphoric acid and phosphate. The experiments were carried out under the most careful control of the dietary intake.

5. In addition, the effect of a very high meat diet (lean beef) was also studied in four other adult men who were members of the technical staff (27, 28, 38 and 43 years of age) The daily food intake was measured roughly, and the variations were somewhat larger than in the phosphorus experiments.

It was calculated that the intake of protein was approximately 70—100 g daily during the control period. The protein intake was raised to 200—230 g daily during the seven days of experiment. The calculated increase in the acidity of the diet was approximately 1200—1400 ml N/10 acid daily.

MATERIAL AND METHODS

Healthy male prisoners, 20 to 56 years of age, have volunteered in the experiments reported. All had been in the prison for some months before joining the balance studies. They had been under careful medical supervision. Blood and urine analyses and, in most cases, X-ray of the whole skeleton, established them as being normal, well-fed individuals. No ill effects have been observed during the experimental periods, although the highest load (3.16 g daily) of orthophosphoric acid, administered thrice daily as reagent grade orthophosphoric acid (84 per cent) in 100 g of apple jam, was felt as a strain.

The four members of the technical staff participating in the high meat diet-experiment (800 g of lean beef daily for seven days) tolerated the high protein diet well.

DIET

The experimental diet for the prisoners has been prepared by an expert dietician, and is weighed and served under strict control. The diet is a good mixed diet. One hot meal is served daily, the menu runs on a 14 day schedule through all experimental and control periods. The bread is a standard rye bread (80 per cent extraction), practically phytin-free. Skimmed milk is the main calcium supply. On the average the daily intake of protein has been 110 g. Foodstuffs rich in phytic acid or oxalic acid have been carefully avoided.

Aliquots of the food as served have been analysed for calcium and phosphorus throughout the experimental periods. All excreta have been collected and analysed through 14 day periods.

ANALYTICAL METHODS

Calcium and phosphorus determined on wet digested material (nitric acid-perchloric acid). Calcium as oxalate by macro and micro permanganate titration, phosphorus photometrically after color development with molybdate and aminonaphtolsulfonic acid.

pH in 24 hour urine, (preserved with thymol) by glass electrode.

Titrable acidity in urine by electrometric titration to pH 7.4.

Preformed urinary ammonia in 24 hour urine by Conway microdiffusion procedure.

Total base in urine by the method of A. Folling (Skand. Arch. f. Physiol. 1931, 21:27) slightly modified.

Potassium in urine by flame photometry.

CO_2-combining capacity of plasma by the Van Slyke manometric gas apparatus.

Unknowns included in all analytical procedures.

Table I. *Calcium in urine in four adult men following ingestion of 2.37 g orthophosphoric acid (750 mg P) daily for seven days. Values expressed as mg Ca/day.*

Exp. person	Control period before ingestion (average of 98 days)		H_3PO_4 (7 days)	Control period after ingestion (average of 42 days)	
	Mean	S. D.		Mean	S. D.
1	107	± 12	87	109	± 11
2	416	± 59	405	465	± 53
3	242	± 33	234	237	± 16
4	412	± 43	337	375	± 22

During H_3PO_4-period: Urinary pH dropped approximately .2—.3 units. Total titrable acidity increased approximately by 150—200 ml n/10 acid. Preformed ammonia increased by approximately 50—100 mg daily. Exp. diet without phosphoric acid gave a urinary pH of 6.0—6.4, and a titrable acidity of 150—250 ml n/10 acid.

Table II. *Calcium in urine in two adult men following ingestion of 3.16 g orthophosphoric acid (1000 mg P) daily for four weeks. Values expressed as mg Ca/day.*

Exp. person	Control period before ingestion (average of 93 days)		H_3PO_4 (28 days)	Control period after ingestion (average of 84 days)	
	Mean	S. D.		Mean	S. D.
2	270	± 5	256	299	± 19
9	212	± 52	167	211	± 26

During H_3PO_4-period: Urinary pH dropped approximately .6—.7 units. Total titrable acidity increased approximately by 200—300 ml n/10 acid. Preformed ammonia increased by approximately 100—300 mg daily.

RESULTS

The results of the experiments with 2.37 g of orthophosphoric acid are presented in Table I. As in the experiments of Farquharson et al. with inorganic phosphate, a slightly diminished output of urinary calcium was observed. The phosphoric acid is excreted in the urine with the aid of some reduction of the pH, an increase in the titrable acidity

Table III. *The calcium balances of two adult men following ingestion of 3.16 g orthophosphoric acid (1000 mg P) daily for four weeks.*

Exp. person	Days	Intake	Urine	Feces	Balances	H_3PO_4 added
		Ca mg/day				
2	98	464	270	153	40	0
	28	423	256	114	53	3.16 g
	84	458	299	102	58	0
9	98	457	212	296	−50	0
	28	431	167	271	− 7	3.16 g
	84	459	211	223	26	0

The P-intake was approximately 1400 mg/day in the control periods, 2400 mg/day in the H_3PO_4-period.

and a slight increase in preformed urinary ammonia. No significant changes in the CO_2-combining capacity of the plasma, indicating metabolic acidosis, were found, nor were significant variations observed in the urinary excretion of potassium and total fixed bases. It should be noted that in P-balance experiments conducted continuously over months in these men, the ingested 750 mg P were recovered in the urine in the course of the experimental week and in the following week. Increasing the daily dose of orthophosphoric acid to 3.16 g for four weeks gave identical changes in the urinary excretion. The pH-lowering effect was slightly more marked, and so was the increase in titrable acidity and ammonia production. The data on urinary excretion and the calcium balances are found in Tables II and III.

The moderate dose of 0.8 g orthophosphoric acid given daily over 8 weeks had no effect on the calcium balance. The evidence in favor of this conclusion appears from Table IV. It should be noted that the 250 mg

Table IV. *The calcium balances of four adult men in the course of eight weeks in which 0.80 g orthophosphoric acid (250 mg P) was added to the diet daily.*

Exp. person	Days	Intake	Urine	Feces	Balance	H_3PO_4 added
		Ca mg/day				
5	56	612	223	410	−21	0
	56	575	162	392	20	.80 g
	84	575	154	398	23	0
6	56	991	342	557	93	0
	56	965	317	556	82	.80 g
	42	985	295	511	179	0
7	56	620	434	267	−101	0
	56	579	401	237	−59	.80 g
	84	594	406	239	−51	0
8	56	973	229	621	123	0
	56	927	219	639	69	.80 g
	84	933	211	655	67	0

P were recovered in the urine during the experimental period.

In the experiments with 750 and 250 mg P as orthophosphoric acid, very detailed studies were carried out during control and experimental periods. These included blood analyses of calcium, inorganic phosphorus, alkaline phosphatase, hemoglobin, NPN, CO_2-combining capacity and total serum proteins. In the urine the daily excretion of organic acids was also followed as was the nitrogen excretion, but as no significant changes were found in either of the two series of experiments, the analytical program was reduced during the other series here reported (experiments with 1000 mg P as orthophosphoric acid, the neutral phosphate mixture and the high meat diet experiments).

Table V presents the experiments with 600 mg P as neutral phosphate added to the usual diet. In one person the fecal calcium

Table V. *The calcium balances of two adult men following the addition of 600 mg P as neutral phosphate daily to the diet for twelve weeks.*

Exp. person	Days	Intake	Urine	Feces	Balances	Remarks
		Ca mg/day				
2	98	465	263	150	52	Average of 98 days, no P added
	84	448	205	146	96	Average of 84 days, 600 mg P added
	112	455	290	113	52	Average of 112 days, no P added
5	98	558	206	418	− 66	No P added
	84	543	173	478	−108	600 mg P added
	112	529	168	621	−260	No P added

The P-intake in the control periods was approximately 1400 mg/day, and 2000 mg/day in the P-period.

is slightly increased, in the other person it is slightly reduced. In both men, some reduction of the urinary calcium occurred during the phosphate period. The Ca-balances to be calculated from the given figures indicate that the balance was not influenced.

Table VI gives the results of the experiments with the high meat diet. Only one of the four experimental persons showed a definite increase in the output of calcium in the urine.

DISCUSSION

The results here presented must be discussed in relation to: 1. The pathways of calcium in the body, and 2. the experiments by previous authors who have produced a distinct effect of acids or acid producing salts on the calcium excretion in the urine.

The one recognized effect of acid or acid salts on calcium in the intestine is

Table VI. *The urinary Ca as influenced by 800 g of lean beef daily in four adult men.*

Exp. person	A	B	C	D	
		Ca mg/day			
	444	121	238	344	pre-control period (6 days)
	352	162	245	363	
	405	127	248	365	
	430	126	282	346	
	372	106	282	363	
	435	136	260	309	
Average:	406	130	263	348	
	461	126	326	308	meat period (7 days)
	388	92	343	436	
	420	133	293	393	
	382	169	180	536	
	430	143	185	354	
	339	186	238	418	
	444	190	190	515	
Average:	409	148	252	423	
	436	93	661	291	post-control period (6 days)
	336	137	222	298	
	253	128	290	286	
	305	176	263	295	
	330	137	225	190	
	315	121	130	259	
Average:	329	132	299	270	

to increase absorption. It is now practically universally recognized that the body regulates its content of calcium only by the way of excretion in the urine. Anions which react with calcium to produce insoluble calcium salts may depress the absorption of calcium. Typical examples are oxalic acid and phytic acid.

As mentioned in the introduction, the results obtained by previous workers with phophates have varied. The results here presented are in agreement with the early work of Farquharson et al., but at variance with

the recent results of Leichsenring et al. (1951). These authors claim that one week is an adequate period for adjustment to a Ca intake as low as 300 mg. Data from the literature (Bauer, Albright & Aub, 1929) furnish support for this claim. The present author has recalculated the data on the normal controls of Bauer et al. together with the data given in their compilation of the literature. This recalculation has been made in order to obtain the variations in daily fecal calcium from one 6-day period to another. Only consecutive 3-day periods have been used. The result of the recalculation shows that of the 15 individuals with two to five consecutive 6-day periods on calcium intakes from 80 to 315 mg daily, 12 showed a small difference between the lowest and the highest daily fecal calcium, viz. less than 70 mg. Three individuals, however, showed a range of 110, 165, and 278 mg respectively. The one with a range of 110 mg in daily fecal calcium will be found in the paper by Sherman, Gillett & Pope (1918). The remaining two with a range of 165 and 278 mg respectively, are normal controls of Bauer et al.

It thus seems well substantiated that on very low calcium intakes, the majority of normal individuals will adapt themselves in the course of about one week and show rather small variations in fecal calcium.

However, experiments of the present author with higher calcium intakes in adult men, followed over many months, have shown that a sudden drop in daily calcium intake from say 900 mg to 500 mg and, on the other hand, a sudden increase of the same order of magnitude, may give very different responses in the fecal calcium in different individuals. In some, adjustment takes place during a 14 day period, in others it may take several weeks. As in our experiments the changes in calcium intake are brought about by altering the daily ration of skimmed milk, a corresponding drop in phosphorus intake will necessarily follow reduction of the calcium intake.

The saturation of the body with calcium, or in other words the daily need for calcium, no doubt plays a major role in determining the length of the adjustment-period.

There is however another observation, reappearing rather frequently in our material, which must be considered in all experiments on calcium metabolism. In the course of experiments where individuals have been followed on the same level of calcium and phosphorus intake for many months, periodical variations in fecal calcium with only small changes in urinary calcium have been observed. These "waves" in the fecal calcium excretion seem in our experiments not to be related to season or vitamin-D fluctuations in the diet, but must at the present time remain unexplained.

As technical errors can be excluded in our experiments these periodical variations over weeks and months in the fecal calcium, must be a reality and cannot be disregarded in further studies on calcium metabolism. The practical consequence of these observations for all studies of calcium requirement, and for the individual reaction to sudden changes in calcium intake, is that conclusions based on brief (one or a few weeks) experiments must be drawn with modesty and great care.

In balance experiments where new variables are suddenly introduced, say by altering the calcium or the phosphorus intake, or both, definite conclusions are permissible

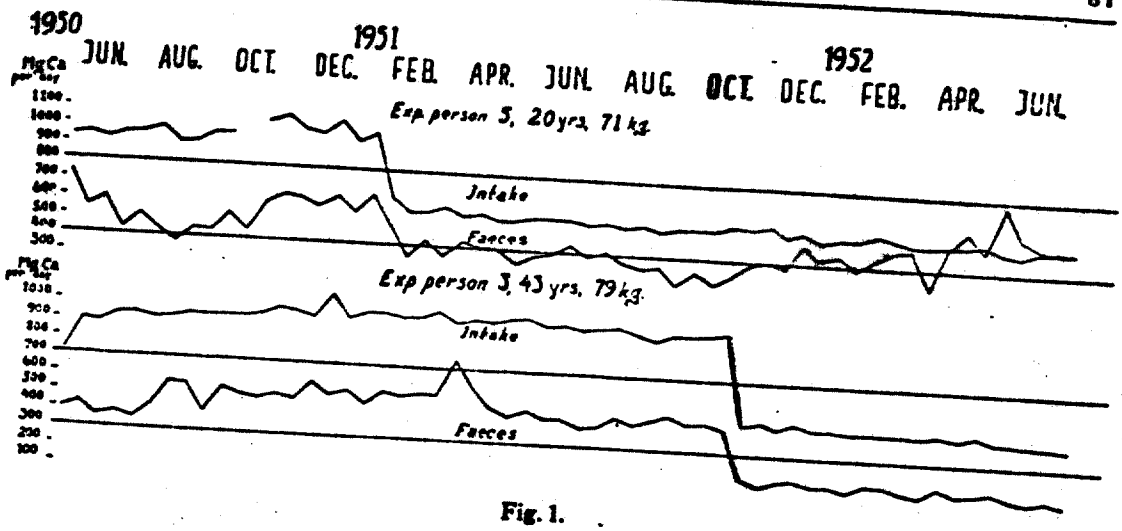

Fig. 1.

only when the individuals in question have been observed for considerable time(months) on a constant diet which will disclose individual periodicity in the fecal calcium in many normal adult individuals. It is then possible to take into account these periodical changes in calcium absorption when conclusions are drawn. In brief experiments on previously unobserved persons one cannot be safeguarded against this capricious individuality in calcium absorption which might lead investigators to erroneous conclusions.

In order to illustrate this individual periodicity in fecal calcium excretion on constant intakes of calcium and phosphorus, data for calcium intake and fecal calcium are given in Fig. 1. Two adult active men, living under the same conditions and on the same experimental diet, have been followed for 24 and 22 months respectively.

It will be noted that when the experiment was started with exp. person No. 3 (43 years of age), significant variations in fecal calcium were observed on a liberal calcium intake (900 mg daily). After 16 months on the same diet, his milk intake was reduced. He adjusted himself during a 14 day period to the lower calcium intake and the fecal calcium showed only small variations during the next 8 months of experiment. This individual is therefore well suited for experiments with variations of phosphorus intake, the calcium intake remaining constant. The other type, which in our material is by no means rare, is represented by exp. person No. 5 (20 years of age). Large variations in fecal calcium were observed when he was put on a liberal calcium intake, the high calcium absorption during the first 5 months of observation indicating a real need for calcium. When his milk intake was reduced, he showed during the following 16 months marked periodical "waves" in the fecal calcium. These variations must make him less well suited for brief experiments, as it seems a matter of chance only whether short time balance studies would find him in a "high fecal calcium phase" or a phase with more efficient calcium absorption.

The observation of such periodical waves in fecal calcium excretion in certain individuals has been taken into account in our interpretations of the experiments reported. Considering next the experiments of Leichsenring et al., it must be questioned if it is warranted to draw the conclusions set forth in their paper. The authors take it for granted that when their experimental individuals adjust themselves over one week to an intake level of 300 mg Ca, they will necessarily do so when the intake is raised to 1500 mg. The opinion is ventured that unless they could demonstrate by protracted studies that their experimental individuals showed only small individual changes in fecal calcium on a high calcium intake (1500 mg), it is unsafe to conclude that the changes observed in fecal calcium are due to different levels of phosphate only. Spontaneous variations in calcium absorption not related to different intakes of phosphates over shorter periods, is another possible explanation which cannot be overlooked.

Turning next to the experiments with phosphoric acid, it follows from the above that the essential problem here is the urinary calcium.

It should be recorded that ammonium chloride, when it increases the calcium excretion in the urine, does so very rapidly. A marked increase is observed within 24 hours.

In the experiments of Farquharson et al., 100 m.e. ammonium chloride increased the urinary calcium by 200—300 per cent.

In the experiments with 24 m Mol. (2.37 g) orthophosphoric acid here presented, the result of one weeks experiment showed a decrease in urinary calcium in all four experimental persons. The decrease was slight in three cases, marked in one.

Experiments carried out later with 32 m Mol. (3.16 g) of orthophosphoric acid over four weeks gave a definite decrease in urinary calcium in both experimental persons.

The results with the high meat diet were mostly negative. It should be noted that the calcium intake in these individuals was comparatively high, and also that the experiments were conducted in healthy and physically active males, in contrast to the hospitalized patients used by Farquharson et al. The striking increase in urinary calcium following immobilization in bed is well known.

The protracted experiments with the 0.8 g orthophosphoric acid in the four men are quite conclusive as to the complete lack of effect of this moderate load over 8 weeks time.

In the relevant series Leichsenring et al. studied the effect of phosphate on a diet containing 1.5 g calcium and 0.8 and 1.4 g P respectively. The extra P was ingested as di-basic calcium and sodium phosphate. They compared the results of four week experiments in two different groups of six persons each. The group on 0.8 g P absorbed on an average 200 mg Ca more than the group on 1.4 g P.

The general experience in our protracted studies on active adult men is that the fecal calcium can vary considerably both from one 2-week period to the next and in longer waves lasting for several 2-week periods.

Recently a very thorough study of the problem has been published by Bonting (1952). He used very high doses of orthophosphoric acid in rats over generations. The results were entirely negative. No effect on

Ca-metabolism or the ash content of the bones was observed. Although care should be taken in applying results from the ingestion of given quantities of acids in rats to man, it is of interest to note the striking similarity between man and rat in their tolerance to phosphoric acid.

Recent experiments on rats in this laboratory (Nicolaysen & Mortenson-Egnund, 1952) strongly support the conclusion that wide variations in the phosphate intake do not affect the absorption of calcium. Two groups of twelve rats each were fed for four successive weeks on a diet with 0.5 per cent calcium. The phosphorus content was 0.34 and 0.57 per cent respectively, achieved by addition of neutral phosphate to the low-P diet. The results were most consistent and showed the same absorption of calcium on different levels of P-intake. The experiments were performed in young vitamin D -free rats which absorbed about 30 per cent of the calcium ingested.

SUMMARY

1. 2.37 g H_3PO_4 (750 mg P) given daily for one week to four adult men engaged in active work, gave a slight decrease in urinary calcium in three cases, and a marked decrease in one case.
2. 3.16 g H_3PO_4 (1000 mg P) given daily for four weeks to two other adults living under the same well-controlled conditions, reduced the urinary calcium slightly in the one, somewhat more markedly in the other case. The calcium balance remained unchanged by phosphoric acid administration in both cases. The calcium intake was about 450 mg daily.
3. 0.8 g H_3PO_4 (250 mg P) given daily for eight weeks to four other adult men under the same experimental conditions had no effect on the calcium balances. The calcium intake was about 950 mg daily in two cases, about 600 mg in the remaining two.
4. An increase of the P-level of the diet from 1.4 to 2.0 g of two active adult men (Ca-intake 0.5 to 0.6 g daily) had no effect on the calcium balances over a period of twelve weeks. The additional 600 mg P were given as a neutral Na-K phosphate mixture. A definite decrease in urinary calcium was observed during the P-period in both cases.
5. Increase of the P-content of the diet by adding orthophosphoric acid or neutral phosphate was followed by a reduction of urinary calcium in all experimental persons.
6. An increase (by lean beef) in the protein intake in the daily diet of four active adult men from approximately 70—100 g to 200—300 g for seven days was followed by a 20 per cent increase of the urinary calcium in one person, by somewhat slighter increase in a second person. In the other two no significant increase occurred.

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BIOCHEMISTRY

Behaviour of Phosphocreatine, Glycogen, and Lactic Acid in Muscles after Sodium Phosphate Administration

by

L. MARKIEWICZ and W. A. ROGOZKIN

Presented by J. HELLER on June 30, 1959

Oral as well as parenteral administration of phosphates prior to physical effort increases efficiency of the organism. This fact, reported by many authors, has not yet been interpreted in terms of biochemistry. Embden et al. [4] believe there is increased phosphorylation of glucose in the muscles, whereas Danilov et al. [3] and Ryabushinskaya [7] think there is stimulation of the central nervous system. Hence, we became interested in investigating the effects of phosphates on the level of phosphocreatine, one of the main sources of muscular energy. Furthermore, the study of the glycogen and lactic acid content in the muscles may give a general idea of the intensity of glycolytic processes.

Methods

The experiments were carried out on white rats weighing 130—170 g. each, divided into two groups. Animals of the first group received intraperitoneally an aqueous solution of NaH_2PO_4 in doses equivalent to 80 mg. of phosphorus (P) per 1 kg. of body weight. The other group served as control and received intraperitoneally 0.9% NaCl in volumes equivalent to those of NaH_2PO_4. After an interval of 30 min., 1, 2, 3, 6 and 24 hours, the animals were decapitated. The gastrocnemius was frozen with liquid oxygen and ground to powder in which phosphocreatine was determined after Aleksieyeva [1], glycogen after Pflüger, and lactic acid after Barker and Summerson [2].

Furthermore, the effects of phosphate administration under conditions of general amytal anaesthesia and/or sciaticotomy were studied in three series. The animals involved in this series of experiments were killed 2 hours after injection of phosphate.

Results

Controls run with animals at rest and injected with physiological saline showed that the level of the compounds in question was in all the series within the limits accepted as normal for rats in this laboratory and

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given by Yakovlev [5]. The level of phosphocreatine was in our experiments 211 mg%, of glycogen 513 mg%, and of lactic acid 53.3 mg% (see Table). These results show that 0.9% NaCl has no visible effect on the content of the three compounds. Sodium phosphate administration, on the other hand, does modify the level of these substances, the effect being dependent on the interval between administration and decapitation. Thus, the level of phosphocreatine falls within thirty minutes by 35 per cent (Fig. 1). It needs to be emphasized that this fall is not caused by

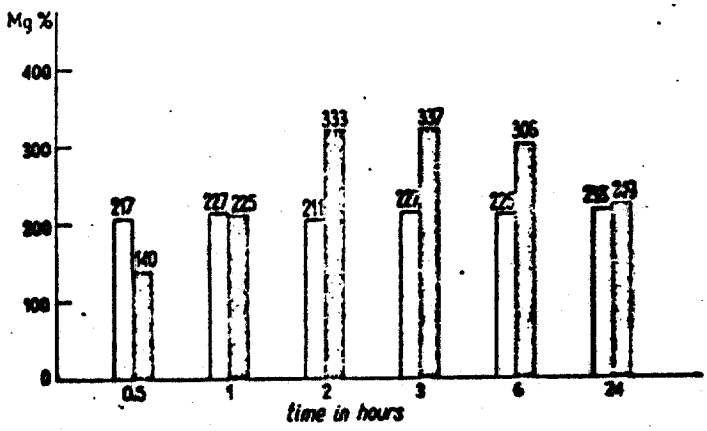

Fig. 1. Content of phosphocreatine in muscles
□ — after administration of 0.9% NaCl
■ — after administration of 30 mg./kg. of NaH_2PO_4

increased motor activity of the animals, since the rats were resting at that time and their behaviour was very much like that of controls. Within 60 minutes the level of phosphocreatine returned to normal, and within 2 hours it was 58 per cent above the original value. Subsequently there is a gradual fall, but the level is after 6 hours still 36 per cent above the initial value. After twenty-four hours, the level is the same as in controls.

The behaviour of glycogen was much less conspicuous, and only the animals killed 2 hours after phosphate administration showed a rise of 18 per cent (Fig. 2). In the remaining series the differences were not statistically significant. It should be noted that in Yakovlev's experiments [5], concerned with the effects of phosphates on glycogen and phosphohexose contents, maximum effects were recorded after an interval of two hours.

In the initial (30 and 60-minute) periods, the level of lactic acid (Fig. 3) rises by 58 to 61 per cent in comparison to controls, and returns then to normal, or slightly below.

The above results suggest increased glycolysis, shortly after phosphate administration, which becomes manifest in increased lactic acid content in muscles. Simultaneously, the level of phosphocreatine becomes depressed without any changes in glycogen content.

Most interesting of all is the considerable increase in phosphocreatine reserves between the second and third hours, which may be responsible for the organism's greater efficiency in physical effort.

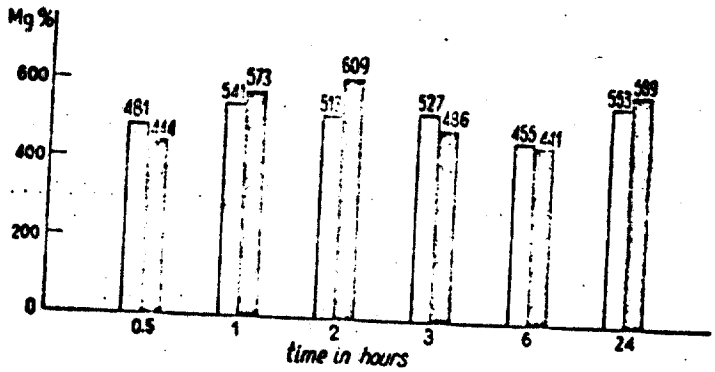

Fig. 2. Content of glycogen in muscles. Explanations as in Fig. 1

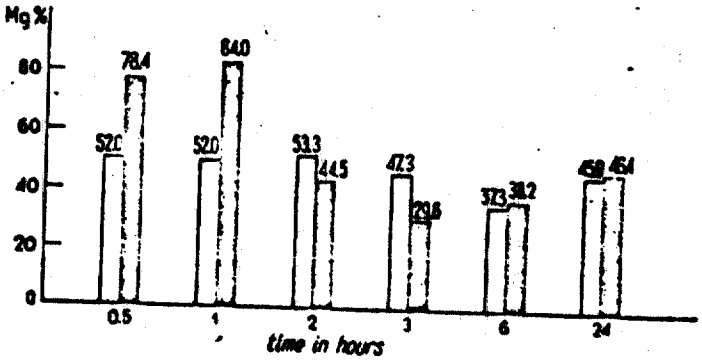

Fig. 3. Content of lactic acid in muscles. Explanations as in Fig. 1

It became, therefore, necessary to test whether this effect involves direct action of phosphate on muscle metabolism, or whether it is attributable to action on the central nervous system. With this in view, a series of experiments was run with animals under deep anaesthesia and with the sciatic nerve cut. The rats were killed two hours after phosphate administration, since this was the interval after which the highest level of phosphocreatine and glycogen was noted in the muscles. Changes in the content of the substances in question (recorded in the Table) were essentially the same as in the previous experiments. The level of phosphocreatine rose by 50—55 per cent, whereas those of glycogen and lactic acid were similar as in controls.

This shows that the phosphocreatine level is increased in muscles irrespective of elimination of the somatic nervous system and that the action of sodium phosphate is, therefore, direct in nature. Indeed, effects of the autonomic nervous system have not been eliminated, but their role does not appear to be of paramount importance, as may be judged from

TABLE

Content of phosphocreatine, glycogen, and lactic acid (in mg%) in muscles two hours after injection

Experimental conditions	Phosphocreatine	Glycogen	Lactic acid
Administration of 0.9% NaCl	211 ± 24.4*)	513 ± 62.0	53.3 ± 7.3
Administration of sodium phosphate (50 mg/kg.)	333 ± 17.8	609 ± 27.6	44.5 ± 3.9
Administration of P under amytal anaesthesia	328 ± 17.6	602 ± 23.1	49.0 ± 8.0
Administration of P under amytal anaesthesia and sciaticotomy	322 ± 15.4	543 ± 17.0	41.2 ± 3.2
Administration of P after sciaticotomy	360 ± 14.6	556 ± 28.1	46.8 ± 6.7

*) — value of arithmetic mean; ± standard error.

Veselkina's experiments [8], who found trophic control of muscular metabolism by somatic nerves. Furthermore, Yakovlev's experiments *in vitro* with muscles [6] showed conspicuous changes in phosphoro-carbohydrate metabolism when phosphorus concentration in the medium was increased.

In the muscles of white rats only 30—40 per cent of total creatine is combined with phosphorus. Administration of phosphates upsets the balance between creatine and phosphocreatine and causes accumulation of the latter, which may be responsible for increased efficiency of the organism in physical effort.

One of the authors (L. M.) carried out the present common work while on leave of absence from the Department of Physiology of the Institute of Physical Culture, Warsaw.

DEPARTMENT OF BIOCHEMISTRY, LENINGRAD SCIENTIFIC RESEARCH INSTITUTE OF PHYSICAL CULTURE

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EXCRETION OF P^{32} IN THE BILE OF RABBITS FOLLOWING INTRAVENOUS ADMINISTRATION*

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The rate of excretion into the bile of certain organic compounds, particularly dyes, has been reported.[1] Similar data on the appearance in the bile of radioactive substances given intravenously are not available. The present study was undertaken to determine the rate of removal of P^{32} from the blood serum and the rate of its appearance in the bile following intravenous administration.

Method. Adult male rabbits were kept under continuous ether anesthesia. A glass cannula was tied into the common bile duct and connected by a rubber tube to a 1 ml. serologic pipet and the abdomen was closed. After the flow of bile was established, 1 ml. of phosphoric acid† containing 2.5 to 3 μc of P^{32} was injected into a vein of the left ear. Samples of blood were taken at intervals from the marginal vein of the right ear. All the bile was collected from the common bile duct in successive portions. Two hours after the injection, the contents of the gallbladder were removed and a sample of blood was obtained from the inferior vena cava. The radioactivity of all samples was then measured, using a thin mica end-window Geiger-Mueller tube. The results obtained were corrected for background and decay. The data were calculated on the basis of an injection of P^{32} equivalent to 1,000,000 counts per minute per kilogram of body weight.

Results. The radioactivity in the blood serum reached a maximum within about 10 minutes following the injection, and that in the bile from the common bile duct, within about 15 minutes. After the maxima were attained, the activities in both serum and bile decreased logarithmically and at about the same rate. As shown in Table 1, the bile from the gallbladder was intermediate in activity between the bile from the duct at the maximum and at the end of two hours in 7 of the 8 rabbits. The activity in the serum at two hours was always higher than that of either the bile from the duct or the bile from the gallbladder at the same time. The ratio of the activity in the serum to that in the bile, as calculated for each sample collected, was fairly constant for each animal. These ratios, as shown in Table 1, were between 5 and 21.

The results in 2 of the rabbits are plotted in Fig. 1. Each point in the curves represents the average over the period of collection; therefore the maximum activity in the bile from the duct in a given period does not appear. As shown by

* Published with permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or conclusions drawn by the authors.

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† Obtained from the Oak Ridge National Laboratories on allocation from the Isotopes Division, U. S. Atomic Energy Commission.

RABBIT	SERUM
1*	1
2*	2
3*	1
4*	1
6	[illegible]
7	18
8	2
9	5

* Actual values, not
cpm signifies cou

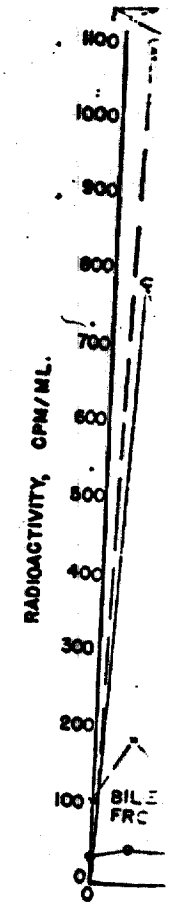

FIG. 1. Activity

TABLE 1

ACTIVITY OF P^{32} IN SERUM AND BILE

RABBIT	SERUM AT 2 HRS.	BILE FROM DUCT		BILE FROM GALLBLADDER AT 2 HRS.	AVERAGE SERUM/BILE RATIO
		*max.	at 2 hrs.		
	cpm/ml.	*cpm/ml.*	*cpm/ml.*	*cpm/ml.*	
1*	136	110	24	106	—
2*	299	392	59	297	—
3*	190	43	61	38	—
4*	140	27	7	40	20.5
6	84	97	7	63	5.4
7	187	189	35	51	5.6
8	242	45	12	35	20.8
9	546	330	72	420	5.2

* Actual values, not calculated, to a dose of 1,000,000 counts per minute per Kg.
cpm signifies counts per minute.

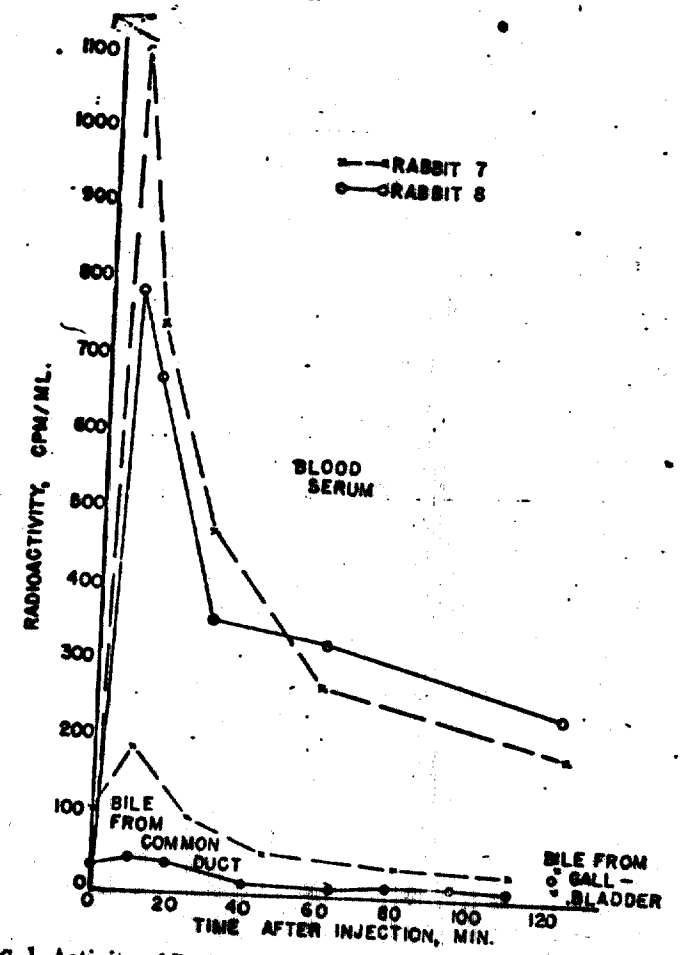

FIG. 1. Activity of P^{32} in the serum and in the bile of 2 of the rabbits.

the curves, the rate of decrease in activity in the bile from the maximum value paralleled that of the serum and the activity in the bile from the duct was continually less than that in the blood serum.

Comment. The experiments demonstrate that transfer of tagged phosphorus from the blood serum into the bile is almost immediate. A ratio of concentration was established within 15 minutes that was maintained for the period of the experiment, even though the P^{32} content of the serum continually decreased. The phosphorus appeared in the gallbladder during the experimental period of two hours in a concentration compatible with previous findings that the gallbladder resorbs approximately half the volume of its contents per hour.[2]

Summary. Following intravenous administration of $H_3P^{32}O_4$ into rabbits, the radioactivity in the blood serum reached a maximum within about 10 minutes, and that in the bile from the common bile duct within about 15 minutes. The rate of decrease in activity in the bile from the maximum value paralleled that of the serum. The activity of the serum was 5 to 21 times that of the bile. Bile from the gallbladder was intermediate in activity between the bile from the duct at the maximum and at the end of two hours.

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CYTOGENETICAL EFFECTS OF INORGANIC PYROPHOSPHATE AND 5-FLUORO DEOXYURIDINE

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I. INTRODUCTION

IN a previous communication (AHNSTRÖM and NATARAJAN, 1965) some aspects of the mechanism of chromosome breakage by chemicals, of the type which inhibits the availability of precursors to the synthesis of DNA were discussed. It was pointed out that the available data indicate that this chromosome breaking effect cannot be solely explained as a consequence of inhibition of DNA synthesis, but may implicate a breakdown of the already synthesized DNA, a mechanism similar to the one operating in the UV irradiated bacteria. It was also indicated by us that this breakdown could be due to the reversal of the action of the enzyme DNA polymerase, caused by an unbalance of the available nucleotide triphosphates necessary for the forward reaction. Among the experimental evidences provided in that study, a chromosome breaking effect of inorganic pyrophosphate (PP) was reported. In our later experiments as well as those of KIHLMAN *et al.* (1966) the chromosome breaking effect of pyrophosphate was not reproducible; hence we undertook a series of experiments to elucidate the conditions under which PP is most effective. Oxygen tension during treatment seems to be an important criterion in manifesting the biological effects of PP in *Vicia faba* cells—low oxygen tensions potentiating the lethal effects of PP.

Since it is known that chromosome breakage; recombination, phage induction in bacteria and breakage of DNA strands *in vivo* are related phenomena and are usually induced by one and the same agent e.g., 5-Fluoro deoxyuridine (FUdR), we undertook a study of the biological effects of PP in diverse systems—such as mitotic recombination in *Ustilago maydis*, lethal effects *in vitro* and *in vivo* of ascites tumour

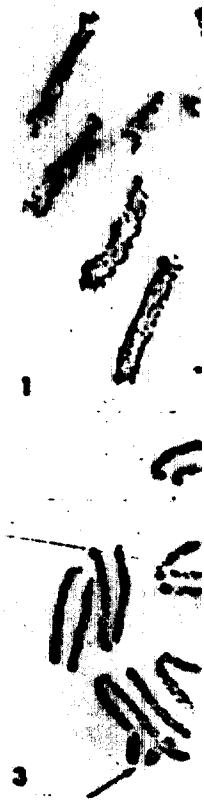
cells (LÖFROTH *et al.*, 1967) and breakdown of DNA in bacteria. All these experiments have indicated a similar end effect between the treatment with FUdR and PP. These results are presented and briefly discussed in this communication.

Material and methods

Secondary roots of *Vicia faba* (var. Weibulls Åkerbröna) were developed by the routine procedure (NATARAJAN and RAO, 1964). Sodium pyrophosphate solution buffered to pH 7.0 was used in all experiments. The secondary roots were developed under well aerated conditions. Three different oxygen levels i.e., 1, 3 and 20 % were used during the treatment. Prior to the treatment the seedlings were equilibrated with the specific O_2 level for 1 hour. The different oxygen concentrations were obtained by mixing air and nitrogen in appropriate proportions. Cytological preparations were made from colchicine pretreated (0.1 % for 2 hrs.) and acetic alcohol (1:3) fixed material processed as Feulgen squashes. In the experiments designed to study the effect of PP on the synthesis of DNA, tritiated thymidine (H^3TdR obtained from Radiochemical Center, Amersham, England, specific activity 6.0 C/mM) was used and the autoradiographs were prepared by coating with Kodak NTB-2 emulsion. After a week's exposure in the dark, the autoradiographs were developed with Kodak 19 B.

Recombination experiments with *Ustilago maydis* were carried out with the diploid H strain (for details, see HOLLIDAY, 1964). This strain in addition to other markers is heterozygous for pfp^2 (resistance to the amino acid analogue—para fluorophenylalanine). Mitotic crossing over can be detected by plating the cells on a medium containing pfp. Mitotic crossing over between the centromere and pfp^1 should produce homozygosity for recessive marker in 50 % of the cases and the recombinants will grow in the selective medium. The results presented in this communication are the average of three experiments and an effective treatment with FUdR was also included in this study for comparison.

Extent of DNA strand breaks was measured as viscosity changes in the DNA following treatment with the agent under study. MENNIGMANN and SZYBALSKI (1962) demonstrated such a breakdown of DNA following treatment of the cells of *Bacillus subtilis* with FUdR, and in their study the fall in viscosity of the DNA could be correlated with the parallel changes in the transforming activity of the DNA. To check whether a similar breakdown of the DNA occurs following PP treat-



Figs. 1—4. — Fig
Fig. 3. Chromatic
region (arrow) ar

Figs. 1—4. — Figs. 1 and 2. Eroded and despiralised chromosomes following treatment with PP. — Fig. 3. Chromatid breaks induced by PP. The breaks are localized at the secondary constriction region (arrow) and the breaks are lying close to the sister chromatids or homologous chromosomes. — Fig. 4. Extreme fragmentation induced by PP.

Figs. 5—8. — Figs. 5 and 6. Chromatid exchanges induced by PP. The exchanges are between homologous chromosomes at homologous sites in Fig. 5 and probably so in Fig. 6. (arrows). — Figs. 7 and 8. Labelling pattern in control (Fig. 8) and PP treated root meristematic cells (Fig. 7) under anaerobic conditions. For details of the experiment, see Table 3. Note the intensively labelled cells in Fig. 8.

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ment, *Escherichia coli* cells were treated with 0.1 M PP solution for two hrs. In a parallel experiment 6×10^{-3} M solution of FUdR for two hrs. was used. DNA from the treated bacteria was isolated, purified and the viscosity measurements were made with an Oswald viscosimeter.

II. RESULTS

1. Cytological studies with Vicia faba

In our earlier study (AHNSTRÖM and NATARAJAN, 1965), a high chromosome breaking ability of PP was reported. In our later experiments as well as those of KIHLMAN *et al.* (1966), these results could not be confirmed. In these experiments, the PP treatment was performed under well aerated conditions. Under such conditions, a high degree of phosphorylation and production of nucleotide triphosphates are expected and this process would be so high that the PP concentration which would inhibit (or revert) the DNA synthesis could not be reached with a nontoxic level of PP. In our earlier experiments, we had used secondary roots developed from seedlings which were not specially aerated (except a change of water every day) and our later experiments were conducted with secondary roots developed under standard conditions with continuous aeration by bubbling air all the time in the culture vessel. When such roots, developed under well aerated conditions were used, very little chromosome breaking effect of PP was found. While high concentrations such as 0.05 M of PP for 4 hrs. and above killed the cells, the lower concentrations did not induce any chromosome aberrations. However, chromosome breaks induced by PP were recovered in cells of roots developed and treated under anaerobic conditions. In order to define the conditions under which PP is effective treatments were given under three oxygen levels i.e., 1, 3 and 20 %. Results of a typical experiment at different oxygen tensions in relation to the induction of chromosome breakage by PP are presented in Table 1. The types of aberrations induced included, gaps, chromatid and iso-chromatid breaks as well as shattering of chromosomes (Figs. 3 and 4). In roots treated with PP (0.002 M) for 4 hrs. and recovered for 18 hrs. a low frequency of chromatid exchanges was observed (4 exchanges in 212 cells scored) (Figs. 5 and 6). These slides were characterized by the absence of anaphases (in roots not pretreated with colchicine), while some immediate effects such as chromatid gaps and breaks were still observable in scorable metaphases. The absence of

TABLE 1. *Effect of PP on Vicia faba cells under different oxygen levels.*
(Treated for 5 hrs. and recovered 90 mins. in 0.1 % colchicine.)

Percent oxygen	Molarity of PP solution	Number of cells with chromosome/chromatid			Number of cells scored
		gaps	breaks	shattered	
20 %	control	0	0	0	100
	0.005	15	1	0	100
	0.002	0	0	0	100
3 %	control	1	0	0	100
	0.005	22	5	7	100
	0.002	0	0	0	100
1 %	control	3	0	0	100
	0.006	(lethal)			
	0.004	(lethal)			
	0.002	14	12	11	131

anaphases may indicate an effect of PP on mitosis in general and spindle in particular.

Other cytological changes induced by PP included pycnosis of nuclei, stickiness of the chromosomes, contraction of the nuclei and chromosomes. A profound effect was also observed on the cytoplasm which tended to break into pieces rather than spread during squashing of the root tips, especially under acute treatment conditions. PP has also an effect on the cell walls; this was specially evident in experiments with Chinese hamster cells in culture, in which the cells were found to get detached from the surface of the glass slips following treatment with PP.

Apart from these cytological effects, the killing effect of PP was also found to increase with lower oxygen tensions. A 0.005 M PP treatment for 4 hrs. could kill the cells of *Vicia* under anaerobic conditions.

A parallel experiment with FUdR was conducted under different oxygen tensions. These results (Table 2) are in direct contrast to those of PP experiments, higher oxygen tension being more effective than the lower oxygen tensions in the production of chromosome breaks by FUdR.

TABLE 2. *Effect of FUdR on Vicia faba cells under different oxygen levels.*

(FUdR- 10^{-6}M treated for 5 hrs. recovered 90 mins. in 0.1 % colchicine.)

Percent of oxygen	No. of cells with chromosome/chromatid			No. of cells scored
	gaps	breaks	shattered	
20	67	32	10	185
3	31	4	2	139

2. Effect of PP on DNA synthesis

In order to asses the effect of PP on the DNA synthesis, a set of experiments was conducted. In the first experiment, aerobically growing *Vicia* roots were pulse labelled with H^3TdR for 30 mts. (6 μc/ml.) followed by a treatment of 0.0025 M PP for 4 hrs. A parallel experiment was done in which the roots were treated with PP (0.0025 M) for 4 hrs. followed by a labelling with H^3TdR (6 μc/ml.) for 90 mts. The rationale of these experiments was to ascertain whether the PP treated cells differ in their capacity to synthesise new DNA (or utilise H^3TdR) from that of the controls. In the first experiment, the frequency of labelled cells was 10.8 % in contrast to 58 % in the controls. The labelling in the PP treated cells was to the extent of 6 to 12 grains/nucleus in contrast to the uncountable number of grains per nucleus in the controls. Cells treated with PP prior to labelling were almost completely devoid of any incorporation of H^3TdR. The few nuclei which showed some labelling had 4 to 8 grains. These results indicate that 4 hrs. treatment of PP considerably alter the DNA synthesising ability of these cells.

In order to assess the influence of PP on the DNA synthesizing capacity of cells under different oxygen tensions (20 and 1 %) an experiment involving these combinations was performed. The details of the experiments are given under Table 3 where the data of these experiments are presented. These data indicate the following: (1) a 2 hr. treatment of PP does not alter the mitotic index under aerobic or anaerobic conditions. (2) The percentage of labelled cells is reduced in PP+H^3TdR treatments significantly from the controls. (3) The percentage of reduction of highly labelled nuclei is maximum under anaerobic conditions (Figs. 7 and 8). (4) The intensity of labelling in-

TABLE 3. *Effect of PP on DNA synthesis as revealed by incorporation of H^3TdR.*

(Roots were treated for 2 hrs. either in H^3TdR alone or H^3TdR+PP (to make a final concentration of 0.002 M.PP) followed by a chase of 10^{-4}M TdR for 45 mins.)

Percent oyygen	Treatment	Mitotic index	% of interphases labelled (A)	Intensively labelled cells (percent of A)
20 %	H^3TdR	8.4	55.7±3.0	15.4±1.5
	H^3TdR+PP	7.4	33.0±2.2	10.0±0.8
1 %	H^3TdR	6.7	54.8±3.9	10.0±1.2
	H^3TdR+PP	6.9	40.6±1.9	2.0±0.25

Nuclei having at least 8 grains over them were counted as labelled. Nuclei having grains that were overlapping and uncountable were classed as intensively labelled cells (Fig. 8). Four random fields in each of five different slides, each slide made by squashing of two root tips, were counted in every case; this amounted to the extent of 1500 to 2000 cells for each treatment.

creases in the presence of oxygen (in controls) as would be expected from the increased phosphorylation. With the method of scoring adopted, the significant point which emerges from this study is that the cells with extreme DNA synthesising activity (as revealed by highly labelled nuclei) are reduced in number by PP under anaerobic conditions. Such highly labelled cells should represent the cells in the 'S' phase (synthetic phase) during the 2 hr. treatment. This reduction indicates that PP has a profound effect on the cells which are in the 'S' stage of the interphase.

3. Detection of PP-induced strand breaks in DNA

The results of the experiments conducted to check whether PP induces strand breaks in DNA leading to fall in viscosity are presented in Table 4. The data indicate that PP induces a breakdown of DNA in bacteria, comparable to an effective treatment with FUdR. The reduction in viscosity induced by PP as well as FUdR are significant (Table 4). The viscocity of DNA following treatment with PP does not significantly differ from the viscocity of DNA following FUdR treatment. The viscosity changes have been interpreted by MENNIGMANN and SZYBALSKI (1962) as due to single strand breaks in the DNA, which after the isolation procedures adopted show up as double strand breaks.

TABLE 4. *Viscosity changes induced in DNA by treatment with PP or FUdR.*

(Average of 3 experiments).

Treatment	Viscosity	Percent of control
Control	0.118±0.0062	100
PP (0.1 M. 2 hrs.)	0.085±0.0082	72.0
FUdR (5.10^{-4}M. 2 hrs.)	0.080±0.0035	67.6

4. Induction of mitotic recombination in Ustilago

The results of the recombination experiment are presented in Figure 9. It can be seen that the recombination rate increases with the increasing concentration of PP up to 0.05 M and then levels off. The increase in the frequency of recombination by PP is significantly higher than the spontaneous rate, and is comparable to the frequency induced by an effective dose of FUdR.

III. DISCUSSION

The data presented here demonstrate that the ability of PP to induce chromosome beraks in *Vicia faba* is a function of the oxidative metabolism in the cells. This could simply be explained by assuming that under anaerobic conditions, the production of deoxyribonucleoside triphosphates is low, and hence, the equilibrium expressed by the equation

$$\begin{matrix} \text{dATP} \\ \text{dGTP} \\ \text{dCTP} \\ \text{TTP} \end{matrix} \underset{\text{DNA polymerase}}{\overset{\text{primer DNA}}{\rightleftharpoons}} \text{DNA} + 4\ \text{PP}$$

is unfavourable for DNA polymerisation. When the cells are aerated, a high oxidative phosphorylation increases the store of triphosphates and consequently, a higher PP concentration is needed to balance this. Finally a concentration is reached where PP will interact not only with DNA synthesis but also with other synthetic processes as well, thus leading to more unspecific effects as obtained in the aerated systems which are described below. FUdR, on the other hand is only effective in well aerated cells, as expected, due to the fact that this compound

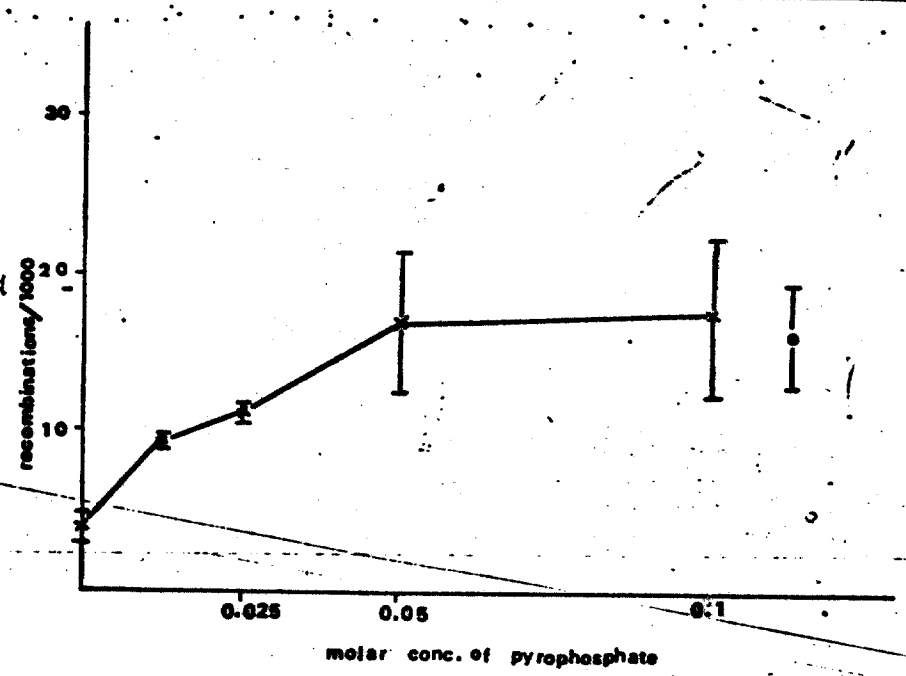

Fig. 9. Frequency of mitotic crossing over induced by PP (×) and FUdR (●). Concentration of FUdR: 10^{-4} M. Treatment for 60 mins.

has to be phosphorylated to inhibit the formation of TTP by inhibiting methylation of dUMP. (See also KIHLMAN, 1962.)

Apart from chromosome breakage, PP induces several other cytological changes which may indicate an effect on the RNA synthesis as well. Pycnosis, stickiness erosion and despiralisation of the chromosomes (Figs. 1 and 2), inhibition of anaphase movement, etc., belong to such a category. In view of this, it is difficult to attribute the effects of PP only to its influence on DNA synthesis. Its chromosome breaking effect however, implicates the involvement of the DNA. With regard to the mechanism of chromosome breakage by PP, nothing new can be added than what has been proposed in our earlier paper (AHNSTRÖM and NATARAJAN, 1965). Studies on the effect of PP on the DNA synthesis, indicate that its presence retards DNA synthesis and very significantly so, under anaerobic conditions. It is hard, however, to demonstrate a breakdown of DNA by autoradiographic techniques. The level at which PP inhibits DNA synthesis is also difficult to answer from the autoradiographic studies.

Studies in *E. coli* have demonstrated that PP can induce a signifi-

cantly high frequency of DNA strand breaks. This end result is similar to that described for FUdR by MENNIGMANN and SZYBALSKI (1962).

The observation that PP induces a significantly high frequency of mitotic recombination is interesting. PP has also been shown to increase meiotic crossing over in *Drosophila* (SHARMA, unpublished). The parallelism between chromosome breakage and crossing over in contrast to mutations in higher organisms has been discussed by several authors (NATARAJAN, 1966). Some aspects of the molecular mechanisms of these processes are considered below.

Mechanism of chromosome breakage and crossing over

The occurrence of chromosome breaks following an inhibition of DNA synthesis has usually been explained as a result of an incomplete DNA synthesis during the late stages of the duplication of chromosomes. We have postulated earlier (AHNSTRÖM and NATARAJAN, 1965) that this type of chromosome breaks could be the result of a real breakdown of DNA and we suggested that the inhibition of precursor formation (or the presence of high PP concentration), might revert the DNA polymerization, where the DNA polymerase could act as a pyrophosphorylytic enzyme. Chemical evidence for this type of reaction has been presented by RICHARDSON *et al.* (1964).

Studies on Ultra-violet irradiated bacteria have shown that the DNA double helix in its non-replicating (resting) form does not seem to be as static as believed earlier and that extensive breakdown and resynthesis of the damaged parts of DNA takes place after the bacterial cells have been exposed to agents such as UV, X-rays and alkylating agents. The repair complex seems to be capable of detecting structural alterations in the DNA helix resulting in an exchange of the damaged part. From several standpoints, crossing over and development of chromosome aberrations seem to involve similar mechanisms i.e., breakage and rejoining of strands. It has also been shown that the ability to repair damaged DNA in many cases parallels with higher frequencies of crossing over—e.g., recombination deficient strain and radio-resistant strains of bacteria (RÖRSCH *et al.*, 1966). The enzyme complex capable of performing this double task i.e., breakage and rejoining, is generally visualised as a polymerase-nuclease complex.

It seems obvious that for an efficient crossing over, a close interaction between the two DNA strands of the homologous chromosomes is needed at the site where crossing over should take place. (Till we know

more about the structure of chromosomes in higher forms, it is practical to assume that each chromatid in a chromosome is made of a long continuous stretch of a DNA double helix, folded and/or spiralised highly.) This, probably, happens in meiosis where the homologous chromosomes are paired during zygotene and pachytene stages. Eventhough some data indicate a pairing during interphase in mitotic interphase also (RAO and NATARAJAN, 1967), this pairing is certainly not as efficient as that occurring during meiosis and this fact will necessitate different models for meiotic and mitotic crossing over.

How should one visualise the pairing process? It is difficult to imagine a base pairing of the type existing between two DNA strands to be present between the paired homologous chromosomes all along their length. However, a DNA double helix is a rather stiff molecule and the packing in the form of a chromosome will necessarily cause stress in certain regions leading to hydrogen bond breakage and strand separation—similar to the tertiary structure of DNA depicted by FREESE (1963). The location of these regions would probably be determined by the gross structure of a chromosome, and consequently, should homologous chromosomes have coinciding denatured regions where base pairing between the chromosomes could occur. In these regions crossing over can take place without difficulty. Assuming that pairing and crossing over occur in the denatured regions of the chromosomes, a model for meiotic crossing over is described in Figure 10. During the DNA synthesis, the growing strands could eventually change partners at the intersection in one end of the pairing region. Breakage and repair, then occurs in this point either as a result of an attack of the repair enzymes on the structural defects caused by the crossing over, or the repair is followed by a mechanical rupture of the chromosomal back bone, when the chromosomes separate. This model assumes that (1) the main DNA synthesis takes place prior to pairing of the homologous chromosomes, (2) the homologous chromosomes pair only in short recurrent regions and (3) a small essential DNA synthesis related to crossing over occurs in these regions when the chromosomes are paired. It should be pointed out that up till now the precise nature and sequence of DNA duplication during meiosis is not clear. (For a critical review on the mechanism of crossing over, see WESTERGAARD, 1964). Though TAYLOR and MC MASTER (1954) demonstrated that DNA synthesis occurs in premeiotic interphase, recent studies have indicated (HOTTA *et al.*, 1966) that a small DNA synthesis during zygotene and pachytene occurs as an essential process.

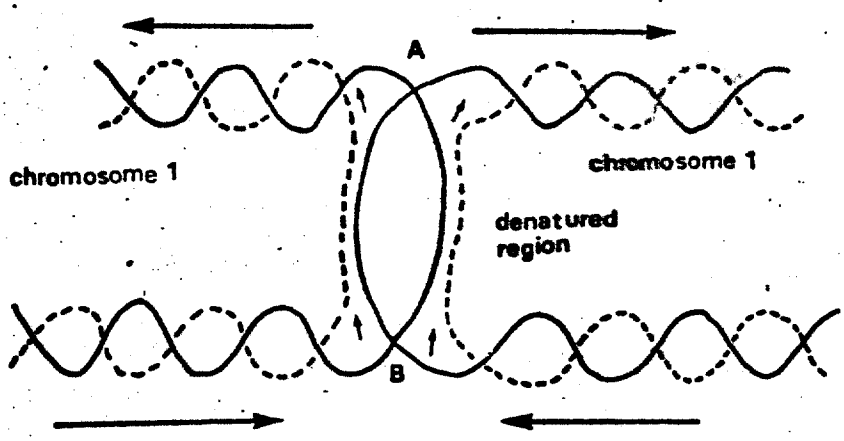

Fig. 10. Model for meiotic crossing over; showing pairing at the denatured regions between the homologous chromosomes 1. Big arrows indicate direction of DNA synthesis. The four small arrows indicate possible "replication forks" where DNA synthesis can start and also the points where new strands can change to the other parent strand. If DNA synthesis starts at a position A, partner change can either take place in a position B (or) in an A position when the new strands from replicon A and B are joined.

The mitotic crossing over is a rare event, when compared to meiotic crossing over, probably due to an incomplete pairing of the homologous chromosomes during the DNA synthesis. The fact that many agents induce somatic crossing over in systems which normally do not show any significant somatic crossing over have tempted several authors to propose that these agents produce a meiosis like condition in mitotic cell leading to pairing of the chromosomes (HOLLIDAY, 1964). Cytological studies in higher organisms have indicated that agents like mitomycin C (which is an efficient recombinogen) induce a high frequency of homologous exchanges (COHEN and SHAW, 1964; RAO and NATARAJAN, 1967). A study of the pattern of chromosome breakage by both bifunctional and monofunctional alkylating agents indicated that such high proportion of homologous exchanges is a common feature following treatment, irrespective of the type of alkylating agent used (RAO and NATARAJAN, 1967). PP induced exchanges were also found to be homologous in nature (Figs. 5 and 6). These have led to the idea that somatic pairing is a normal phenomenon in higher organisms (RAO and NATARAJAN, 1967). The mitotic crossing over is probably more dependent on primary single strand breakage and a temperory pairing. The primary single strand breaks are produced as a result of direct

action of the agent in question, X-rays or alkylating agents etc. or produced in an enzymatical process either by repair enzymes attacking structural defects caused by the treatment (UV or alkylating agents) or by a pyrophosphorylatic attack from the DNA polymerase or a special pyrophosphorylatic enzyme, due to an unbalance in the precursor pool. In the later case, the broken ends are also prepared for rejoining by the presence of an energy rich bond in contrast to a situation when a break is introduced by a nuclease attack.

If the assumption that somatic pairing occurs commonly in higher organisms is correct, one may speculate the role of such a pairing. The most possible role seems to be connected with the genetic regulation. It may be stated that in an heterozygote, the dominant gene exerts its influence by inhibiting its homologous recessive. A classical material in which this can be studied is the salivary glands of the dipterian larvae, where the homologous chromosomes in their polytene condition are somatically paired. Several studies made in heterozygotes of diptera such as Chironomids, *Sciara*, have shown that the puffs (an expression of genetic activity) occurs only in one of the chromosomes in the locus in question (see references listed by ASHBURNER, 1967). By this it is not implied that such a simple mechanism will accomodate all aspects of gene action in terms of structural, regulator and operator genes. Secondly, it is not clear as to how in molecular terms one can explain the role of somatic pairing in controlling the expression of recessive genes by the dominant ones. If the occurrence of somatic pairing is universal, this must have a fundamental functional significance and such a control of gene activity seems to be an important consequence.

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SUMMARY

This study has demonstrated that inorganic pyrophosphate induces chromosomal aberrations only under anaerobic conditions in contrast to FUdR, which is most efficient when treated under aerobic conditions. The ability of PP to induce breaks in DNA as well as to induce mitotic recombination in *Ustilago* have been studied and compared with that of FUdR. The mechanisms of chromosome breakage, meiotic recombination and mitotic recombination are briefly discussed.

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ET RÉACTIVATION COMPLÈTES DE LA PHOSPHOMONO-ALINE ET INTERCHANGEABILITÉ DES MÉTAUX ACTIFS

par

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I. INTRODUCTION

monoestérase alcaline (rein, intestin) perd pratiquement toute activité ngée contre des solutions de p_H inférieur à 6.0 (H. ALBERS, BEYER, MÜLLER [1], D. ALBERS [2]) ou contre des milieux neutres renfermant complexe, tels que les cyanures alcalins, le diéthyldithiocarbamate pyridyl (CLOETENS [3], J. ROCHE ET NGUYEN-VAN THOAI [4]). L'enzyme re une partie de son pouvoir déphosphorylant lorsqu'on le met en premier cas de son dialysat, dans le second de certains cations diva- s Mg^{++}, Mn^{++} et Zn^{++}. Les conditions dans lesquelles on observe nt fait considérer l'inactivation et la réactivation de la phosphatase nduisant la dissociation réversible, soit d'une cophosphatase organique t de métaux jouant le rôle de coenzyme [4,6].

ur ces faits, de nombreux auteurs attribuent une fonction coenzy- ions activateurs. Pour eux, diverses phosphatases alcalines sont des renfermant spécifiquement, les unes du magnésium, les autres du se, du cobalt (CLOETENS [3], HOVE, ELVEHJEM ET HART [6], JENNER ET ET DUFAIT [8], MASSART ET VANDENDRIESSCHE [9]). Selon CLOETENS [3], eux métaux, le zinc et le magnésium, serait même indispendable à osphatase rénale. Toutefois, l'argumentation conférant un rôle spécifi- eux métaux en raison de leur aptitude à activer des préparations enzy- ente pas une sécurité absolue, car l'intensité de l'effet exercé par les on du degré de pureté de la phosphatase et son sens peut même s'inver- es conditions (NGUYEN-VAN THOAI [10], NGUYEN-VAN THOAI ET RAY- urs, de nombreux auteurs, entre autres BAMANN, HEUMÜLLER, WERNER RE ET NGUYEN-VAN THOAI ([4] et travaux non publiés), ont observé ations divalents, dont Mg^{++}, Mn^{++}, et Zn^{++} sont en général les aptes à renforcer ou à restaurer l'activité phosphatasique, à des con- culières à chacun. Aussi peut-on se demander si-et dans quelle mesure- présenter une spécificité étroite comme constituant de la phosphatase ane et d'une humeur déterminés, ou si certains éléments divalents, magnésium, le manganèse, le zinc et, accessoirement, le calcium et le à cet égard interchangeables.

ation des faits observés demeure hypothétique. En effet, d'une part, ble de préciser si les produits réactivant la phosphatase alcaline renfer- me ou des effecteurs et, d'autre part, la répétition de certains travaux

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antérieurs conduit à des résultats parfois contradictoires *. Il en est sans doute ainsi pour deux raisons, à savoir : l'emploi fréquent de préparations insuffisamment purifiées, non comparables entre elles et renfermant en proportions diverses des constituants susceptibles de former des complexes avec les métaux, et, par ailleurs, la méconnaissance des conditions absolues d'activation et de réactivation de la phosphatase alcaline par des cations, conditions ne pouvant être définies qu'avec des produits purs ou hautement purifiés.

Nous nous sommes proposés d'entreprendre l'étude de la constitution de la phosphomonoestérase alcaline en mettant à profit les résultats de nos recherches antérieures sur la purification de cet enzyme [13] et sur son activation par des cations divalents en présence d'acides aminés [5] ; ce mémoire expose la première partie de notre travail. L'élimination d'impuretés métalliques au cours de la préparation de produits purifiés et l'activation de ceux-ci par des cations à concentration optima couplés à des acides aminés formant avec eux des complexes, nous ont en effet paru être des conditions en dehors desquelles une telle étude ne saurait donner que des résultats incertains. Il convenait en effet, à la fois d'éliminer des impuretés susceptibles de troubler la réactivation et d'assurer celle-ci au degré le plus élevé, ce qui n'est possible qu'en présence de cations et d'un acide aminé. Enfin, il était important de réaliser une inactivation et une réactivation *complètes* de la phosphatase pour discuter le mécanisme de ces processus, afin de distinguer l'action d'effecteurs et celle de produits susceptibles de participer à la structure de l'enzyme. Or, les recherches antérieures sur la reconstruction de celui-ci à l'aide d'un coenzyme hypothétique, dissociable en milieu faiblement acide, ont montré que l'on ne restaure que 20 à 25 pour 100 de l'activité initiale si la dissociation préalable de la phosphatase a été pratiquement totale, le retour au pouvoir phosphoestérasique primitif n'étant observé que si la dialyse à $p_H = 6.0$ n'a pas réduit celui-ci de plus de 60 à 70 pour 100 (D. ALBERS [2]). Quant aux réactivations par des cations, elles n'ont en général été précédées que d'une disparition partielle de la propriété déphosphorylante des milieux étudiés, due parfois à la présence de formateurs de complexes jouant le rôle d'inhibiteurs (voir note ci-dessous).

Ceci étant, nous avons considéré que l'on obtiendrait des données utiles sur le rôle et la spécificité des métaux divalents en réalisant l'inactivation complète de la phosphatase par dissociation d'un coenzyme organique hypothétique ou d'un métal et sa réactivation par ceux-ci. Nous nous sommes adressés pour obtenir la première à la dialyse prolongée à 37° contre de l'eau bidistillée, et pour assurer la seconde à l'addition de divers cations en présence d'alanine.

Le but de ce travail est :

1°. de préciser les conditions d'inactivation totale par dialyse prolongée contre

* Les expériences ayant amené CLOETENS [3] à conclure que la phosphatase alcaline du rein renferme nécessairement du zinc et du magnésium comme métaux actifs sont basées sur le fait qu'après dialyse contre des solutions de cyanure de potassium, puis de bicarbonate de sodium, l'addition d'un sel de magnésium ne réactive l'enzyme que si elle a été précédée de celle d'un sel de zinc. Reprenant ces essais, nous avons constaté qu'il n'en est pas ainsi lorsque des préparations purifiées (fractionnement acétonique des préparations obtenues par la technique de D. ALBERS [14]) de l'enzyme sont inactivées par dialyse prolongée contre des solutions de cyanures alcalins (6 jours à 37°), puis de l'eau bidistillée (15 à 20 jours à 37°). L'addition d'ions Mg^{++} permet alors à elle seule de réactiver au maximum la phosphatase et il est probable que l'effet de l'ion Zn^{++} dans les conditions adoptées par CLOETENS est dû à l'élimination incomplète du radical—CN, inhibiteur de l'enzyme, Zn^{++} intervenant alors comme antiinhibiteur.

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de l'eau bidistillée de la phosphatase alcaline de l'intestin purifiée et celles de sa réactivation complète par des cations divalents ;

2°. de démontrer l'interchangeabilité des cations divalents dans cet enzyme ;

3°. de discuter le rôle coenzymatique éventuel d'un métal et le mécanisme de sa participation aux processus d'inactivation et de réactivation étudiés.

2. PARTIE EXPÉRIMENTALE

a. *Matériel d'étude et techniques.* La phosphatase alcaline a été préparée par une technique dérivant de celle permettant d'obtenir l'enzyme cristallisé (NGUYEN-VAN THOAI, ROCHE ET SARTORI [13]), à partir des muqueuses intestinales de Chien, matériel beaucoup plus actif que celui provenant des animaux d'abattoir. 0,5 kilogramme de tissu non autolysé, prélevé par raclage au scalpel de la surface interne de l'intestin, est mis en suspension dans 1,0 litre d'eau bidistillée renfermant 25 pour 100 d'acétone pure et additionné de 100 cc d'un mélange à parties égales d'acétate d'éthyle et de toluène (H. ALBERS ET E. ALBERS). Après deux jours de séjour à température du laboratoire en agitant deux à trois fois par jour, on filtre sur papier et on refroidit progressivement à —2° environ. On ajoute par petites portions de l'acétone pure, fraîchement distillée, refroidie à —5° jusqu'à ce que le milieu en renferme 60 pour 100. Après séjour d'un quart d'heure dans un bain à —5°, on siphonne le liquide au-dessous duquel s'est déposé un précipité blanc, que l'on isole par centrifugation. Ce précipité est lavé trois fois à l'acétone pure, anhydre, refroidie à —5° et séché dans le vide ; il constitue une préparation de phosphatase brute, susceptible d'être conservée plusieurs mois sous vide. Le produit ainsi obtenu est partiellement soluble dans l'eau ; on en prépare un extrait en le triturant au mortier avec 20 fois son poids d'eau bidistillée, puis on complète à 50 volumes de celle-ci. Après filtration du résidu insoluble (mucoprotéines dénaturées), la solution aqueuse est portée à 0°, puis fractionnée par addition d'acétone pure refroidie à —5°, introduite dans le milieu par petites portions et en agitant le récipient placé dans un bain à —5°. La fraction protéique précipitant à la concentration de 38 pour 100 en acétone, pauvre en enzyme et riche en impuretés, est rejetée, tandis que l'on conserve celle précipitant aux concentrations comprises entre 38 et 55 pour 100 en acétone *. Ces dernières sont séparées par centrifugation et l'on chasse l'acétone en faisant passer un courant d'air rapide à la surface des culots protéiques refroidis à —5°, lesquels renferment la plus grande partie de la phosphatase et de petites quantités de mucoprotéine. Au lieu de procéder à la cristallisation de la première, opération qui comporte des pertes d'activité non négligeables à partir de ce matériel, nous avons traité par l'eau bidistillée les précipités obtenus et utilisé leurs solutions aqueuses à 0,5—1,0 pour 100. Nos expériences ont donc été poursuivies sur une phosphatase alcaline d'un assez haut degré de pureté, les fractions du produit brut solubles dans les conditions indiquées étant en général susceptibles de permettre la cristallisation en masse de l'enzyme.

L'activité phosphatasique des solutions non dialysées a été déterminée sur 0,2 cc de celles-ci en présence de 4 cc de solution tampon de MICHAËLIS au véronal sodique de p_H = 8,8, de 2 cc de solution M/10 de β-glycérophosphate de sodium et de 3,8 cc d'eau bidistillée, toutes les solutions étant préparées avec cette dernière. L'essai, dont le p_H est égal à 8,8—8,9, est maintenu à 37° pendant 5 minutes, le récipient et les solutions étant portés à cette température avant leur emploi. L'addition d'ions Mg^{++} s'est avérée inutile pour activer l'enzyme, probablement en raison de la saturation de celui-ci en métal. L'hydrolyse du substrat est arrêtée au moyen de 0,5 cc d'acide sulfurique à 30 pour 100 et les radicaux phosphoriques libérés sont dosés par colorimétrie (méthode de BRIGGS-ROBISON avec lectures au photomètre de Pulfrich, en utilisant le filtre S[57]).

b. *Inactivation et réactivation complètes de la phosphatase alcaline.* L'inactivation totale de la phosphatase alcaline a été réalisée par dialyse prolongée contre de l'eau

* Dans les extraits aqueux d'organes frais, la phosphatase alcaline précipite à une teneur sensiblement plus élevée en acétone ; nous reviendrons sur la signification de ce fait.

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bidistillée. Cette opération a été poursuivie à 37° en présence de toluène, jusqu'à disparition de tout pouvoir phosphomonoestérasique, ce qui exige environ vingt jours, le liquide dans lequel le sac à dialyse est immergé étant renouvelé tous les deux jours. Il est indispensable de suivre par de fréquents dosages la diminution de l'activité enzymatique, surtout dans la période terminale de l'expérience, car le processus étudié n'est réversible qu'à condition de ne pas opérer sur un produit totalement inactivé depuis plus de 24 heures.

Quant à la réactivation par les métaux divalents en présence d'alanine, elle s'est révélée possible à un degré élevé uniquement après une incubation préalable de l'enzyme additionné de l'acide aminé à $p_H = 8,8$, comme tel est également le cas lors du „transport de la cophosphatase" [1] et de la réactivation par l'ion Mn^{++} de la *l*-leucylpeptidase intestinale (SMITH ET BERGMANN [15]), de l'arginase hépatique (HUNTER ET DOWNS [16], MOHAMED ET GREENBERG [17]) et de la carboxylase de levure (GREEN, HERBERT ET SUBRAHMANYAN [18]). Les métaux divalents mis en oeuvre dans réaction ont été apportés par des solutions d'acétate de magnésium ou de calcium et de sulfate de fer (ferreux), de manganèse ou de zinc M/10—M/1000 ajoutées à des taux correspondant à ceux indiqués dans les tableaux ci-dessous (concentrations optima ou voisines déterminées au cours de nos recherches antérieures).

Le dosage de l'activité phosphatasique des produits ainsi régénérés a été opéré de la manière suivante : 0,2 cc de solution enzymatique ont été additionnés de 4 cc de solution tampon de MICHAËLIS au véronal sodique de $p_H = 9,8$ de 0,2 cc de solution M/1 de *dl*-alanine ajustée à $p_H = 8,8$ et de 2,7 cc d'eau bidistillée et placés pendant deux heures à 37°, puis mélangés à 2 cc de solution m/10 de β-glycérophosphate de sodium et complétés à 10 cc avec de l'eau bidistillée. Après 5 minutes de séjour à 37°, la réaction enzymatique est arrêtée par l'addition de 0,5 cc d'acide sulfurique à 30 pour 100 et les radicaux phosphoriques libérés sont dosés par colorimétrie.

On trouvera ci-dessous les résultats d'une de nos expériences, cités à titre d'exemple et exprimés en gamma de phosphore libérés par minute et par milligramme d'azote protéique présent dans la préparation enzymatique étudiée.

Les faits qui se dégagent des données obtenues sont les suivants : la phosphatase dialysée longuement à 37° contre de l'eau bidistillée n'hydrolyse plus le ß-glycérophosphate de sodium. L'addition d'ions Mg^{++} et, à un degré moindre, celle d'ions Mn^{++} en présence d'alanine restaure partiellement l'activité enzymatique initiale, Zn^{++}, Ca^{++} et Fe^{++} étant à cet égard inefficaces. Cette réactivation est plus faible que celle observée dans nos expériences précédentes, faites sur des enzymes moins purifiés et n'ayant pas subi une aussi longue dialyse. Elle est spécifique du pouvoir phosphomonoestérasique, car elle ne va de pair avec l'apparition d'aucune activité pyrophosphatasique. Dans les conditions de nos essais actuels, l'*incubation préalable en présence d'alanine à $p_H = 8,8$ et à 37° est indispensable pour une réactivation importante de la phosphatase par les cations divalents*. En introduisant ceux-ci immédiatement avant le substrat, Ca^{++}, Fe^{++}, Mn^{++}, Zn^{++} sont alors à peu près également activateurs aux concentrations optima indiquées, Mg^{++} étant à cet égard plus efficace. Il a été possible d'hydrolyser en sa présence jusqu'à 700 millimolécules de β-glycérophosphate de sodium par gramme d'azote et par minute à $p_H = 8,8$ et à 37°. Cette activité enzymatique est environ deux fois et demi plus forte que celle de la phosphatase cristal-

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TABLEAU I

RÉACTIVATION DE LA PHOSPHOMONOESTÉRASE ALCALINE (intestin de Chien) TOTALEMENT INACTIVÉE PAR DIALYSE PROLONGÉE CONTRE DE L'EAU BIDISTILLÉE, AU MOYEN DE CATIONS DIVALENTS (Ca^{++}, Fe^{++}, Mg^{++}, Mn^{++} et Zn^{++}) ET EN PRÉSENCE D'ALANINE.

Essais poursuivis avec ou sans incubation de 2 heures à 37° en présence de *dl*-alanine avant l'addition des solutions de sels métalliques. Résultats exprimés en gamma de phosphore minéral libérés par minute et par milligramme d'azote protéique à $p_H = 8,8$ et à 37° aux dépens de ß-glycérophosphate de sodium M/50 dans les conditions décrites section a.

Sel métallique ajouté	Concentration moléculaire en sel	Activité (gamma P libérés/minute/ mgr. N protéique
Série I : en présence d'alanine, mais sans incubation préalable avec celle-ci		
Néant	0	0
SO_4Fe	1.10^{-7} M	0
	1.10^{-5} M	0
SO_4Zn	1.10^{-7} M	0
	1.10^{-6} M	0
SO_4Mn	1.10^{-3} M	721
	1.10^{-2} M	981
$(CH_3.COO)_2Ca$	1.10^{-3} M	0
	1.10^{-2} M	0
$(CH_3.COO)_2Mg$	1.10^{-2} M	288
	5.10^{-2} M	1.154
	1.10^{-1} M	1.635
Série II : incubation en présence d'alanine, suivie de l'addition de sels métalliques		
Néant	0	5.461
SO_4Fe	1.10^{-7} M	14.231
	1.10^{-6} M	13.261
SO_4Zn	1.10^{-7} M	14.231
	1.10^{-6} M	13.261
SO_4Mn	1.10^{-3} M	15.385
	1.10^{-2} M	13.646
$(CH_3.COO)_2Ca$	1.10^{-3} M	14.231
	1.10^{-2} M	16.364
$(CH_3.COO)_2Mg$	1.10^{-2} M	18.654
	5.10^{-2} M	21.154
	1.10^{-1} M	21.154

Activité avant dialyse, sans incubation ni effecteur: 3.336
Activité après dialyse, sans incubation ni effecteur: 0
Activité après dialyse et incubation (2 heures $p_H = 8,8$) sans effecteur: 461

lisée mise en oeuvre en présence de sulfate de magnésium $1—10^{-2}$ M * ; elle est du même ordre de grandeur que celles de la zymohexase du muscle (450 millimol gr de substrat/grN/min) et de l'énolase de levure (1—100 millimol gr de substrat/grN/min) cristallisées par WARBURG ET CHRISTIAN [35, 36]. L'activité la plus élevée des préparations

* Le produit initial mis en oeuvre dans l'exemple cité (Tableau I) est environ 7 fois moins actif que la préparation réactivée au maximum par Mg. Il est impossible de prendre l'activité d'une phosphatase comme test de sa pureté tant que l'on n'est pas certain d'avoir pu la faire agir dans des conditions ou son pouvoir hydrolytique s'exerce au maximum. La définition de ces conditions est l'un des objets même du travail actuel.

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de phosphatase alcaline étudiées exprimée en microtitres de gaz (1 mol d'orthophosphate occupant 22,4 l) libérés pas mgr de substance et par heure Qp, est égalé à 170.000, valeur de Qp très notablement supérieure à celles obtenues jusqu'ici dans le cas du même enzyme purifié par d'autres méthodes [35, 36].

Les cations activateurs peuvent indifféremment être ajoutés avant ou après incubation en présence d'alanine, à l'exception du manganèse dont l'hydrate précipitant à pH = 8.8 gêne la réactivation, probablement en dénaturant l'apoenzyme lorsqu'il est maintenu longuement au contact de celui-ci. A cette exception près, l' *action des cations s'exerce aussi bien, que l'enzyme ait subi ou non un contact prolongé avec eux avant addition du substrat.* Dans les conditions adoptées, Zn^{++} et Fe^{++} exercent leur effet activateur maximum à l'état de traces (1.10^{-7}—1.10^{-6} M), Mn^{++} et Ca^{++} à des taux plus élevés (1.10^{-3}—1.10^{-2} M) et Mg^{++} à forte concentration (1.10^{-2}—1.10^{-1} M). La nécessité d'une concentration très élevée en magnésium pour réactiver la carboxylase (KUBOWITZ ET LUTTGENS [19]), enzyme renfermant naturellement ce métal à très faible taux, mérite d'être rapprochée de nos observations.

Comme dans le cas de l'énolase (WARBURG ET CHRISTIAN [20]) et des peptidases (MASCHMANN [21]), nous avons observé un *seuil* de concentration du magnésium au delà duquel son efficacité n'augmente plus, tandis que, pour les autres métaux étudiés, en particulier pour le fer et le zinc, fortement inhibiteurs à des taux élevés, il existe une *concentration optima.* Dans l'expérience dont les résultats figurent au Tableau 1, la quantité de préparation renfermant 10 γ d'azote, soit au plus 60 γ de protéine, est réactivée au maximum par 12 mgr de magnésium soit près de 200 fois son poids de celui-ci. Une surcharge double en ce métal, soit 24 mgr, conduit à un résultat identique. L'activité maxima particulière aux autres cations, inférieure à celle obtenue avec Mg^{++}, est atteinte à des taux beaucoup moindres de ceux-ci, très différents selon le métal mis en oeuvre : 0,065 γ de zinc ou de fer, 540 γ de manganèse et 4.000 γ de calcium. Devant l'existence d'un seuil de concentration pour l'action maxima du magnésium et d'une concentration optima pour celle du zinc et du manganèse, WARBURG ET CHRISTIAN ont admis que le premier est le constituant minéral naturel de l'énolase.

Nous examinerons plus bas s'il y a lieu d'adopter la même manière de voir en ce qui concerne la phosphatase alcaline. Nous analyserons au cours de la discussion des résultats la part que l'on peut attribuer aux cations et à l'acide aminé dans les phénomènes étudiés, mais il y a lieu de faire dès maintenant certaines remarques. L'incubation à pH = 8,8 de l'enzyme inactivé fait réapparaître à elle seule un faible pouvoir phosphoestérasique (voir Tableau I, témoin 2) et celui-ci est assez important si l'opération a lieu en présence d'alanine (voir Tableau I, témoin 2). Ces faits ne sont pas de nature à rendre trop incertaine l'interprétation de ceux résumés plus haut. En effet, l'analyse des préparations enzymatiques étudiées nous a montré que celles-ci renferment du magnésium à des taux relativement élevés et de petites quantités de zinc, dont une partie seulement est éliminée par dialyse lorsque les préparations sont totalement inactivées. Il suffit que l'incubation à pH = 8,8 provoque la dissociation d'une faible partie de ces métaux fixés à des impuretés (mucoprotéines) pour que leurs ions réactivent l'enzyme. De même la mise en liberté, à l'état d'ions, de traces indosables de métaux tels que le zinc, dont nous venons de signaler l'élimination incomplète, explique en partie l'effet de l'incubation en présence d'alanine, effet auquel participent également d'autres phénomènes liés à l'état de l'apoenzyme protéique.

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c. Inhibition de la réactivation de la phosphatase alcaline par les fluorures, les phosphates et les pyrophosphates alcalins. La réactivation de la phosphatase alcaline par les cations en présence d'un acide aminé une fois obtenue, nous avons essayé de mettre en concurrence des effecteurs antagonistes, inhibiteurs, et l'acide aminé associé au cation permettant la réactivation la plus forte, le magnésium .En effet, si ces derniers participent à la reconstruction de l'enzyme ou de son groupement actif, la fixation préalable à l'apoenzyme de radicaux susceptibles de s'unir à l'un ou à l'autre doit gêner la réactivation. C'est ainsi que, pour WARBURG ET CHRISTIAN [20], l'ion fluor empêcherait l'activation en donnant naissance à un complexe fluoromagnésien inactif de l'enzyme (Mg—F—énolase).

Nous avons été orientés dans le choix des effecteurs par des expériences préliminaires, au cours desquelles nous avons observé que la phosphatase alcaline purifiée est très sensible à la présence de divers anions aptes à donner des complexes avec les métaux, entre autres les ions fluor, orthophosphorique et pyrophosphorique. Le premier inhibe faiblement et irrégulièrement l'enzyme brut dans les extraits des tissus animaux (BELFANTI, CONTARDI ET ERCOLI [22]; ROCHE, DE LAROMIGUIÈRE ET LAURENS [23]), où le second est toujours inhibiteur (ERDTMANN [24]), le troisième n'ayant pas été étudié à cet égard. Tous trois inhibent énergiquement la phosphatase alcaline lorsque celle-ci a été purifiée et débarassée d'antiinhibiteurs naturels, opération qui exige l'élimination des mucoprotéines présentes dans les préparations enzymatiques pauvres en azote bien qu' assez actives obtenues par SCHMIDT et TANNHAÜSER [35] (10.1% N) et par CAPUTTO ET MARSAL [36] (7-10 % N).

Après dialyse jusqu'à perte pratiquement totale de son pouvoir hydrolytique, la phosphatase est, soit mise pendant 2 heures à l'étuve à 37° et à $p_H = 8,8$ (solution tampon au véronal de MICHAELIS) en présence de fluorure de sodium (1.10^{-3}—5.10^{-2} M), de phosphate disodique (1.10^{-3} M) ou de pyrophosphate de sodium (2.10^{-3}—1.10^{-2} M), puis additionnée de *dl*-alanine (1.10^{-2} M), soit soumise aux mêmes opérations en inversant l'ordre des réactifs, l'incubation ayant lieu en présence de l'acide aminé et les formateurs de complexe étant ajoutés par la suite ; de l'acétate de magnésium (1.10^{-2}—5.10^{-2} M) est introduit immédiatement avant le substrat. L'activité de ces milieux vis-à-vis du ß-glycérophosphate de sodium a été déterminée dans les mêmes conditions qu'au cours des expériences décrites dans le paragraphe précédent, chaque essai renfermant 0.2 cc de solution enzymatique, 4 cc de solution tampon au véronal sodique de MICHAELIS de $p_H = 8,8$ 2 cc de ß-glycérophosphate de sodium M/10 et 3,8 cc d'eau bidistillée ou des réactifs affecteurs énumérés ci-dessus. On trouvera dans le Tableau II quelques uns de nos résultats.

Les résultats obtenus peuvent être résumés de la manière suivante : L'incubation de la phosphatase alcaline en présence de fluorure de sodium réduit fortement l'effet de l'ion magnésium et cela que de l'alanine, activatrice par elle-même en l'absence de ce sel soit (II*b*) ou non (I*b*) ajoutée après cette opération. Lorsque du phosphate disodique (III*b* et *c*) ou du pyrophosphate de sodium (IV *a* et *b*) est substitué au fluorure alcalin, l'activation de l'enzyme par l'ion magnésium en présence d'alanine est nulle dans le premier cas et faible dans le second *. *Les anions étudiés empêchent donc à divers degrés*

* Les préparations étudiées étant rigoureusement dépourvues d'activité pyrophosphatasique, il est légitime d'attribuer aux ions pyrophosphoriques — et non à des ions orthophosphoriques provenant de leur hydrolyse éventuelle — les effets observés en leur présence.

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TABLEAU II

INFLUENCE DE L'INCUBATION PRÉALABLE (2 heures à 37° et à p_H = 8,8) DE LA PHOSPHATASE ALCALINE (intestin de Chien) EN PRÉSENCE SOIT D'ALANINE, SOIT DE FLUORURE, D'ORTHOPHOSPHATE OU DE PYROPHOSPHATE DE SODIUM SUR L'ACTION ANTAGONISTE DE CES DIVERS EFFECTEURS VIS-A-VIS DE LA RÉACTIVATION DE L'ENZYME PAR L'ION MAGNÉSIUM APRÈS INACTIVATION PRESQUE COMPLETE PAR DIALYSE (15—20 jours à 37°) CONTRE DE L'EAU BIDISTILLÉE

Résultats exprimés en γP minéral (orthophosphates) libérés par minute et par milligramme d'azote protéique aux dépens de β-glycérophosphate de sodium M/50 à 37° et à p_H 8,8 dans les conditions expérimentales décrites Section a.

Préparation et série d'essais N°	Effecteurs mis en incubation	Effecteurs ajoutés après l'incubation	Gamma P minéral libérés par min et mgr N prot.
I *a*	néant	$(CH_3.COO)_2Mg$ 5.10^{-2} M	3.173
I *b*	FNa 1.10^{-2} M	$(CH_3.COO)_2Mg$ 5.10^{-2} M	1.538
I *c*	alanine 1.10^{-2} M	$(CH_3.COO)_2Mg$ 5.10^{-2} M	17.500
I *d*	alanine 1.10^{-2} M	FNa 1.10^{-2} M + $(CH_3.COO)_2Mg$ 5.10^{-2} M	16.346
II *a*	alanine 1.10^{-2} M	$(CH_3.COO)_2Mg$ 5.10^{-2} M	13.796
II *b*	FNa 1.10^{-2} M	alanine 1.10^{-2} M + $(CH_3.COO)_2Mg$ 5.10^{-2} M	6.018
II *c*	alanine 1.10^{-2} M	FNa 1.10^{-2} M + $(CH_3.COO)_2Mg$ 5.10^{-2} M	14.186
II *d*	alanine 1.10^{-2} M	FNa 1.10^{-2} M + $(CH_3.COO)_2Mg$ 5.10^{-2} M	11.722
III *a*	néant	néant	615
III *b*	PO_4Na_2H 1.10^{-2} M	néant	0
III *c*	PO_4Na_2H 1.10^{-2} M + $(CH_3.COO)_2Mg$ 1.10^{-2} M	néant	1800
III *d*	alanine 1.10^{-2} M	néant	3.953
III *e*	alanine 1.10^{-2} M	PO_4Na_2H 1.10^{-2} M	3.715
III *f*	alanine 1.10^{-2} M	PO_4Na_2H 1.10^{-2} M + $(CH_3.COO)_2Mg$ 1.10^{-2} M	7.323
IV *a*	$P_2O_7Na_2$ 5.10^{-3} M	alanine 1.10^{-2} M + $(CH_3.COO)_2Mg$ 5.10^{-3} M	2.629
IV *b*	$P_2O_7Na_2$ 1.10^{-2} M	alanine 1.10^{-2} M + $(CH_3.COO)_2Mg$ 2.10^{-2} M	629
IV *c*	alanine 1.10^{-2} M	$P_2O_7Na_4$ 2.10^{-3} M + $(CH_3.COO)_2Mg$ 5.10^{-3} M	15.074
IV *d*	alanine 1.10^{-2} M	$P_2O_7Na_4$ 1.10^{-2} M + $(CH_3.COO)_2Mg$ 2.10^{-2} M	13.703

l'acide aminé et l'ion magnésium de réactiver l'enzyme. L'incubation en présence d'alanine réactive par elle-même la phosphatase (Ic et III*d*) et renforce considérablement l'effet de Mg^{++}, comme on l'a vu plus haut (Tableau I), mais l'addition ultérieure de fluorure (I *d*), de phosphate (III *e* et *f*) ou de pyrophosphate de sodium (IV *c* et *d*) ne provoque plus alors qu'une inhibition faible ou nulle et n'empêche pratiquement pas l'activation par le magnésium en présence de l'acide aminé. *L'alanine se comporte donc comme un antiinhibiteur car, après incubation en sa présence, l'enzyme devient très peu sensible aux ions fluor, orthophosphorique et pyrophosphorique.*

3. DISCUSSION

La dialyse prolongée contre de l'eau bidistillée inactive complètement la phosphatase alcaline purifiée. Celle-ci reprend un pouvoir phosphomonoestérasique très élevé après incubation en présence d'alanine à p_H = 8,8 et addition de cations divalents.

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à p_H = 8,8) DE LA PHOSPHATASE
E, SOIT DE FLUORURE, D'ORTHO-
ON ANTAGONISTE DE CES DIVERS
PAR L'ION MAGNÉSIUM APRÈS
jours à 37°) CONTRE DE L'EAU

es) libérés par minute et par
osphate de sodium M/50 à 37°
Section a.

teurs après ation	Gamma P minéral libérés par min et mgr N prot.
Mg 5.10^{-2} M	3.173
Mg 5.10^{-2} M	1.538
Mg 5.10^{-2} M	17.500
$_3$.COO)$_2$Mg 5.10^{-2} M	16.346
Mg 5.10^{-2} M	13.796
$_3$.COO)$_2$Mg 5.10^{-2} M	6.018
COO)$_2$Mg 5.10^{-2} M	14.186
COO)$_2$Mg 5.10^{-2} M	11.722
nt	615
nt	0
nt	1800
nt	3.953
1.10^{-2} M	3.715
$_3$.COO)$_2$Mg 1.10^{-2}M	7.323
$_3$.COO)$_2$Mg 5.10^{-3} M	2.629
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entre autres de Mg++, et une concurrence se manifeste à cet égard entre l'acide aminé, activateur, et les ions fluor, orthophosphorique ou pyrophosphorique, inhibiteurs. Ces faits établis, il convient de les interpréter et d'en discuter la signification en ce qui concerne, d'une part, le mécanisme général de l'inactivation et de la réactivation de l'enzyme et, d'autre part, l'interchangeabilité des cations mis en oeuvre dans celle-ci.

a. *Mécanisme général de l'inactivation et de la réactivation de la phosphatase alcaline.* L'inactivation par dialyse prolongée contre de l'eau bidistillée peut être due soit à la dissociation du groupement actif de l'holoenzyme, soit à la rupture ou à la formation de liaisons analogues à celles maintenant associées les „unités" distinguées par SVEDBERG dans les molécules protéiques. La nécessité absolue de cations divalents pour obtenir la restauration d'un pouvoir phosphatasique très élevé permet de supposer qu'un métal divalent est éliminé au cours de la dialyse ; mais les effets de l'incubation en la présence d'un acide aminé ne sauraient relever de la même explication. *Les phénomènes observés semblent donc être la résultante de plusieurs processus, à savoir : la rétrogradation de modifications de structure de l'apoenzyme et la reconstitution d'un groupement actif métallique ou rattaché à l'apoenzyme par un métal divalent.*

Les molécules protéiques sont susceptibles de se dissocier réversiblement sans rupture de liaisons de valence primaire, mais seulement par celle de liaisons mettant en jeu des valences coordinatives ou des forces de cohésion d'une autre nature (force d'adsorption, forces de VAN DER WAALS en particulier). C'est ainsi que des modifications du p_H au delà de la zone de stabilité (SVEDBERG [25]), l'addition d'urée (STEINHARDT [26]) ou de salicylate de sodium (ANSON ET MIRSKY [27]) à forte concentration, parfois même de faibles différences de salinité (BROHULT [28]), peuvent entrainer la dissociation de protéines de poids moléculaire élevé et, dans certains cas, leur dénaturation. Cette dernière est réversible dans des conditions particulières ; la réaction, dite réversion (ANSON ET MIRSKY), est alors toujours assez lente et provoque la réapparition de certains caractères particuliers à la protéine naturelle. L'aptitude des globines naturelles à se combiner à la protohémine pour donner naissance aux méthémoglobines, la perte de cette propriété lors de la dénaturation et sa restauration après réversion (ANSON ET MIRSKY [29], HILL ET HOLDEN [30], ROCHE ET COMBETTE [31], ROCHE ET CHOUAÏECH [32]) constituent à cet égard un exemple caractéristique. Or, on peut se demander dans quelle mesure la dénaturation de l'apophosphatase n'est pas responsable de la perte de l'activité enzymatique au cours de nos expériences et si l'effet de l'incubation n'est pas lié à un processus de réversion. La lenteur avec laquelle la réactivation est obtenue rappelle celle de la réversion de la globine à $p_H = 6,5$ et, par ailleurs, le mode de préparation de l'enzyme, commandé par la nécessité de le séparer d'impuretés multiples, et sa très longue dialyse à 37° sont susceptibles de le dénaturer.

Le caractère non immédiat de la réactivation de la phosphatase alcaline doit être rapproché de celui que présente également sa reconstitution à partir des produits de sa dissociation („cophosphatase" et „apophosphatase" [1, 5]). Pareil fait n'est d'ailleurs pas exceptionnel en enzymologie ; il a été observé par GREEN, HERBERT ET SUBRAHMANYAN [18] lors de la préparation de la carboxylase à partir de son apoenzyme, d'aneurinediphosphate et de cations divalents, au cours de la réactivation de l'apoenzyme de l'arginase hépatique (HUNTER ET DOWNS [16], MOHAMED ET GREENBERG [17]) et de la *l*-leucylpeptidase intestinale (SMITH ET BERGMANN [15]) par l'ion Mn^{++}. Dans ce dernier cas, un séjour de 24 heures et plus à 37°, dans des conditions p_H bien définies, est nécessaire pour obtenir l'activité enzymatique maxima. La part de la réversion de la dénaturation dans cette

L'hypothèse p. 75.

„time reaction", considérée souvent comme particulière à la chimie des enzymes, nous semble importante et des recherches sont actuellement entreprises pour contrôler cette hypothèse. On peut aisément concevoir que la dénaturation ait fait perdre à l'apoenzyme la propriété de fixer un cation indispensable à son activité et que la réversion provoque la mise en liberté des groupements fonctionnels susceptibles de s'unir spécifiquement au métal *.

Mais, si cette hypothèse de travail mérite d'être retenue, elle ne rend néanmoins compte qu'incomplètement du mécanisme des faits observés. Il est possible que l'acide aminé favorise la réversion en se fixant à certains radicaux de la protéine ou par une réaction d'un autre type, mais le rôle propre des cations et son couplage à celui de l'acide aminé sont, par ailleurs, très importants. L'explication la plus simple que l'on puisse donner des phénomènes auxquels ils participent, inspirée par de multiples observations faites sur d'autres enzymes à métal dissociable, paraît être la suivante. L'inactivation par dialyse prolongée traduirait la perte progressive d'un constituant métallique et il serait alors nécessaire de saturer à nouveau l'enzyme en métal pour qu'il retrouve son activité maxima. L'expérience montre qu'il ne suffit pas pour cela de le mettre en présence de cations ; il convient que ceux-ci soient associés à un acide aminé, divers corps de cette série pouvant être mis en oeuvre dans ce but sans qu'une spécificité se manifeste pour l'un d'entre eux. Pareil rôle coactivateur d'un acide aminé vis-à-vis d'un enzyme purifié a été observé dans d'autres cas, en particulier dans l'activation de l'adénylpyrophosphatase (BAILEY [33]), de diverses peptidases (MASCHMANN [21]) et de la zymohexase de levure (WARBURG ET CHRISTIAN [34]). Son mécanisme a été discuté par ces derniers auteurs, selon lesquels l'acide aminé (cystéine) interviendrait, soit comme régulateur de valence des ions lorsque ceux-ci peuvent exister sous un état di- ou tri-valent ($Fe^{++} \rightleftarrows Fe^{+++} + e^-$) un seul de ceux-ci étant actif, soit comme régulateur de concentration des ions avec lesquels ils forment des complexes. La seconde de ces hypothèses a été adoptée par MASSART ET VANDENDRIESSCHE [9], pour expliquer le rôle du glycocolle dans l'activation de la phosphatase du lait et du rein par l'ion Zn^{++} dans un milieu renfermant cet acide aminé. Ce rôle nous paraît être plus complexe.

En effet, d'une part les acides aminés ont une action propre, et d'autre part ils empêchent les fluorures, les phosphates et les orthophosphates d'exercer leur influence inhibitrice. On admet souvent que leur pouvoir activateur est uniquement lié à l'élimination d'inhibiteurs naturels métalliques constituant des impuretés accompagnant l'enzyme et avec lesquels ils formeraient des complexes; mais ce n'est là sans doute qu'une partie de leur action, car leur antagonisme avec les anions inhibiteurs que nous avons étudié

* La réversion de la dénaturation peut ne porter spécifiquement que sur une partie de ce processus. Dans le cas particulier des globines on restaure bien l'aptitude des protéines dénaturées à se combiner à l'hématine pour donner des méthémoglobines identiques aux produits naturels par leur spectre, leur solubilité, leur poids moléculaire et par diverses autres propriétés, mais le groupement prosthétique ne s'unit alors à la protéine que d'une façon beaucoup plus labile que dans les chromoprotéides naturels (ROCHE ET CHOUAÏECH [32]). S'il en est de même lors de la réversion de la dénaturation des apoenzymes, il est possible que cette réaction ne permette qu'une réactivation incomplète. Le fait que, dans les conditions où nous nous sommes placés, celle-ci conduise à des pouvoirs phosphatasiques plus élevés que ceux des produits cristallisés permet de penser que la cristallisation de la phosphatase va de pair avec un certain degré de dénaturation, comme dans le cas d'autres enzymes (catalase). C'est sans doute au même phénomène qu'il y a lieu de rattacher les pertes d'activité observées après des cristallisations successives [13] et la diminution de solubilité de l'enzyme dans des milieux de teneur croissante en acétone au cours de sa purification.

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sives [18] et la diminution de
e en acétone au cours de sa

ne peut être expliqué ainsi. Cet antagonisme même traduit probablement le fait que les uns et les autres se fixent sur le constituant métallique de l'enzyme, le radical métal-anion (fluorure, phosphate, pyrophosphate) ne participant pas au pouvoir phosphatasique. Nos observations conduisent à se représenter le groupement actif comme un complexe de métal divalent avec certains radicaux de l'apoenzyme et il est possible que l'acide aminé joue un rôle important dans la formation du complexe actif, soit en „transportant" le métal auquel il est lié à des radicaux particuliers de l'apoenzyme, soit en participant à des complexes du type apoenzyme-métal-acide aminé. Enfin, le fait que l'incubation de l'enzyme inactivé doit, pour avoir toute son efficacité, être opérée en la présence d'un acide aminé, permet de penser que ce dernier intervient par lui-même dans la réversion de la dénaturation de l'apoenzyme. Aussi ne saurait-on interpréter simplement la fonction dévolue à l'alanine dans la réactivation de la phosphatase. Pareille complexité s'est manifestée dans le rôle des acides aminés en tant que coactivateurs des ions Ca^{++}, Mn^{++}, vis-à-vis de l'adénylpyrophosphatase (myosine cristallisée) qui, pour BAILEY [33], consisterait en „l'élimination par formation de complexes de métaux lourds présents dans les préparations de myosine, l'augmentation de la stabilité de l'enzyme vis-à-vis de la chaleur et en une action spécifique de l'ion dipolaire".

b. *Interchangeabilité des métaux actifs et constituant métallique naturel.* Dans les conditions où nous nous sommes placés, la réactivation de la phosphatase alcaline est assurée par divers cations divalents en présence d'un acide aminé. Il n'existe à cet égard aucune spécificité étroite du métal, mais seulement des différences dans les concentrations auxquelles chacun exerce sont effet maximum (concentration optima) et dans l'intensité de son action. Une interprétation simple de ce fait peut être donnée. La réactivation comporte nécessairement la combinaison des cations à l'apoenzyme, sans doute par formation de complexes du métal avec des radicaux azotés de la protéine. Or l'affinité de ces radicaux n'est à coup sûr pas identique pour les divers cations et il est logique que le zinc, formant très facilement des complexes avec les groupements aminés des protéines et des peptides, „sature" l'enzyme à très faible concentration, tandis que le magnésium, participant beaucoup plus difficilement à des complexes, ne le ferait qu'à des taux très élevés. Un excès de zinc ou de fer doit par ailleurs se combiner à l'apoenzyme en dehors du groupement actif, surtout après la saturation de celui-ci, et il en résulterait une inhibition non spécifique. Par contre, la faible affinité de la protéine pour le magnésium la rend peu apte à s'unir à celui-ci, lequel n'est inhibiteur à aucune concentration dans les conditions où nous nous sommes placés *. Des faits du même ordre ont été signalés par MASCHMANN [21] à propos de l'action des métaux sur diverses peptidases, Mg^{++} et Mn^{++} présentant alors un seuil de concentration à partir duquel ils exercent un effet maximum (1.10^{-3} M), tandis qu'il existe pour Zn^{++} une concentration optima (1.10^{-7}—10^{-6} M), au delà de laquelle cet ion est fortement

* Dans les produits bruts, l'ion magnésium est faiblement inhibiteur à concentration très élevée (en l'absence d'acide aminé) ; il n'en est pas ainsi dans nos essais, soit en raison du caractère incomplet de la réversion des produits purifiés utilisés, soit, plus simplement, en raison de la présence d'alanine. Par ailleurs, l'activabilité de la phosphatase alcaline par l'ion magnésium est lié au degré de saturation de l'enzyme en métal. C'est ainsi que la phosphatase intestinale partiellement purifiée de SCHMIDT ET TANNHAÜSER [33] est peu sensible à cet ion parce que son métal naturel ne s'est pas dissocié au cours de sa préparation. L'augmentation du pouvoir activateur, des sels de magnésium par dilution et après une brève dialyse des extraits de muqueux intestinale signalée par BACCARI ET AVICENNO [37] peut, par contre être reliée à la dissociation du constituant métallique naturel de la phosphatase.

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inhibiteur. Dès lors, l'argumentation selon laquelle un cation activateur présentant un seuil d'action (Mg) doit plus probablement être considéré comme le constituant naturel d'un enzyme à métal dissociable que ceux (Fe, Zn, Mn) pour lesquels il existe une concentration optima, paraît fragile.

L'interchangeabilité expérimentale des cations divalents pose la question de la spécificité du constituant minéral naturel de la phosphatase alcaline. Le magnésium permet d'atteindre l'activité la plus élevée dans nos expériences, mais seulement à très forte concentration, tandis que le zinc et le fer provoquent une activité moindre, mais très importante, à l'état de traces (1.10^{-7}—1.10^{-6} M). Il ne semble pas possible, en l'état actuel de nos connaissances, d'affirmer que la phosphatase alcaline soit une métalloprotéine renfermant du magnésium dans un organe, du zinc, du fer, du manganèse ou du calcium dans d'autres, la spécificité relative d'activation par l'un de ceux-ci observée par divers auteurs dans des conditions particulières pouvant tenir à la présence d'impuretés. Néanmoins il est probable que seuls le magnésium ou le zinc doivent être considérés comme constituants naturels de la phosphatase alcaline, l'interchangeabilité de l'un et de l'autre pouvant être envisagée. L'étude de la composition des produits purs ou très hautement purifiés extraits de divers organes, actuellement en cours, apportera une réponse ferme à cette question.

La nécessité, admise par CLOETENS [3], de la présence de deux métaux, le magnésium et le zinc, dans la phosphatase alcaline ne semble pas devoir être retenue. Des expériences antérieures nous ont montré que la réactivation par l'ion magnésium n'est nullement renforcée par la présence d' ions zinc et des résultats nouveaux, dont il ne nous a pas paru utile de faire figurer ici le détail, ont permis de constater que Zn^{++} n'est activateur en présence de Mg^{++} que dans la mesure où la concentration de ce dernier est trop faible pour que le pouvoir phosphatasique maximum soit restauré par lui. Il y a alors addition des effets de Mg^{++} et de Zn^{++}, mais non la „potentialisation" de ceux-ci décrite par CLOETENS sur des produits moins purifiés que ceux utilisés pour nos recherches ; l'hypothèse de cet auteur sur la constitution de la phosphatase alcaline doit donc être abandonnée.

Le manque de spécificité des cations activateurs de la phosphatase alcaline rend peu probable la participation directe du métal à l'activité enzymatique. Divers faits établis sur la carboxylase (KUBOWITZ et LÜTTGENS [19] ; GREEN, HEREERT ET SUBRAHMANYAN [21]) sont à rapprocher de ceux décrits dans notre travail. Dans le cas de ce dernier enzyme, le magnésium naturel est remplaçable par le manganèse, le cobalt et par d'autres cations divalents, et le groupement actif serait le radical —NH_2 fixé en position 6 au cycle pyrimidinique de l'aneurine ; le métal constituerait „un ciment, un pont chimique" entre l'apoenzyme et le coenzyme, associés l'un et l'autre dans un complexe magnésien. Aussi s'explique-t-on qu'il n'existe aucun lien étroit entre la nature du cation permettant de reconstituer la carboxylase à partir de ses produits de dissociation organiques (apoenzyme et aneurinediphosphate) et la spécificité de l'enzyme. Seul le degré d'activité du produit obtenu est fonction du métal présent *. Il est légitime d'admettre comme hypothèse de travail qu'il en est de même pour la phosphatase alcaline ; dès lors, le métal de celle-ci ne jouerait pas le rôle de coferment qui lui est souvent attribué, car il ne participerait qu' *indirectement* à l'activité catalytique de l'enzyme.

* Il n'existe pratiquement aucune différence d'activité entre les carboxylases „reconstituées" à Mg ou Mn ; celles renfermant Ca, Co, Fe et surtout Zn sont sensiblement moins actives.

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Les expériences d' H. ALBERS et de ses collaborateurs[1] et les nôtres[5] sur le „transport du coferment" devront être reprises sur des produits purs pour qu'une opinion ferme soit formulée sur ce point, mais leurs résultats et ceux de nos recherches actuelles permettent de se faire une première représentation hypothétique de la molécule enzymatique. Celle-ci paraît être constituée soit par un complexe métallique — en général magnésien ou zincique — de l'apoenzyme et d'un corps organique dissociable, soit par un complexe dont les radicaux organiques spécifiquement unis au métal seraient tous empruntés à l'apoenzyme ; il n' existerait pas alors de coenzyme dissociable. Dans le premier cas, un peptide pourrait constituer le coenzyme et être remplaçable par divers acides aminés ; c'est là une éventualité peu probable. Dans le second, la coactivation par les acides aminés serait due à une sorte d'orientation du métal uni à l'acide aminé vers les groupements de l'apoenzyme aptes à se combiner à lui pour former un complexe actif. Ce dernier ne comprendrait pas nécessairement l'acide aminé, lequel pourrait n'être qu'un simple „transporteur" du métal. Si hypothétique que soit cette interprétation générale, elle nous a paru mériter d'être formulée pour objectiver la notion que le constituant métallique de la phosphatase alcaline (ou de phosphatases alcalines, d'apoenzymes divers) ne doit pas être considéré comme son coenzyme au même titre que le cuivre de certaines polyphénoloxydases et le zinc de l'anhydrase carbonique. Ceux-ci sont rigoureusement spécifiques d'un enzyme ou d'un type d'enzyme et ont une fonction catalytique, tandis que le métal de la phosphatase alcaline, étranger au groupement actif proprement dit, joue aussi — et peut-être exclusivement un — rôle de *structure* compatible avec une spécificité beaucoup moins étroite.

RÉSUMÉ

1. La phosphatase alcaline purifiée (phosphomonoestérase de l'intestin de Chien) a été complètement inactivée par dialyse prolongée contre de l'eau bidistillée et réactivée. Le pouvoir hydrolytique obtenu est supérieur à celui du produit initial, l'enzyme dédoublant alors jusqu'à 700 milimolécules de β-glycérophosphate de sodium par minute et par gramme d'azote, à $p_H = 8{,}8$ et à 37° ($Q_P = 170.000$).

2. La réactivation maxima de la phosphatase a été réalisée après incubation (2 heures) de l'enzyme avec de l'alanine (1.10^{-2} M) à $p_H = 8{,}8$ et addition d' ions magnésium. Dans les mêmes conditions, d'autres cations divalents : Ca^{++}, Fe^{++}, Mn^{++} et Zn^{++}, réactivent l'enzyme à des degrés divers. Il existe pour eux une concentration optima, très faible pour Zn^{++} et Fe^{++} (1.10^{-7} M), plus élevée pour Mn^{++} (1.10^{-3} M) et pour Ca (1.10^{-2} M), tandis que l'on observe un seuil d'action pour Mg^{++}, actif avec son efficacité la plus grande à des taux supérieurs à 1.10^{-2} M.

3. L'incubation préalable avec l'acide aminé est indispensable ; lorsqu'elle n'a pas eu lieu, seuls Mg^{++} et, à un degré moindre, Mn^{++}, sont activateurs, mais beaucoup plus faiblement qu'après séjour à $p_H = 8{,}8$ et à 37° de l'enzyme additionné d'alanine. Il est probable que ces faits sont en partie dus à la réversion de la dénaturation de la phosphatase en présence de l'acide aminé plutôt qu'à la lenteur de la réaction de celui-ci avec l'enzyme. L'incubation avec des ions métalliques est sans efficacité propre, qu'elle soit opérée avant ou après l'addition d'alanine. Tout se passe donc comme si la combinaison du métal à l'enzyme était immédiate.

4. L'incubation préalable de la phosphatase purifiée en présence de fluorures, d'orthophosphates, de pyrophosphates alcalins, empêche la réactivation par les acides aminés et les cations divalents. Inversement, l'incubation préalable de l'enzyme en présence d'alanine gêne très fortement l'action inhibitrice des fluorures, des orthophosphates et des pyrophosphates alcalins. On peut en déduire que les acides aminés et les anions de ces derniers sels se combinent sans doute au même élément structural du groupement actif de la phosphatase.

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5. On ne saurait trop insister sur le fait que l'action des effecteurs, en particulier des cations et des formateurs de complexe, sur la phosphatase alcaline ne s'exerce régulièrement que sur des produits purifiés et à des concentrations en métaux bien définies, différentes pour chacun de ceux-ci. Dans le cas contraire, elle est fonction de la présence de métaux et de formateurs de complexes naturels dans les préparations enzymatiques. Les multiples contradictions entre de nombreux travaux doivent être rattachées à cette cause ; des résultats divers, facilement reproductibles, peuvent en effet être obtenus selon le mode opératoire adopté.

6. La signification de ces faits en ce qui concerne la constitution de la phosphatase alcaline est discutée. L'interchangeabilité des métaux actifs n'est pas favorable à l'hypothèse admettant qu'il existe dans les divers organes des phosphatases dont chacune renferme spécifiquement un métal particulier au tissu ou elle est présente. Le magnésium, et à un degré moindre le zinc, paraît être le constituant minéral le plus commun des phosphatases alcalines et y jouer un rôle d'élément de structure plutôt que celui d'un coenzyme catalytiquement actif.

SUMMARY

1. Purified alkaline phosphatase (phosphomonoesterase from dog's intestine) has been completely inactivated by prolonged dialysis against bidistilled water and reactivated. The hydrolytic power obtained, superior to that of the initial product, attains maximal 700 millimolecules of β-glycerophosphate of sodium per minute and per gramme of nitrogen, at a p_H of 8,8, and at 37° C (Q_p = 170.000).

2. The maximal reactivation of the phosphatase has been observed after incubation for 2 hours of the enzyme with alanine (1.10^{-2}M), at a p_H of 8,8 and addition of magnesium ions. Under the same conditions, other divalent cathions (Ca^{++}, Fe^{++}, Mn^{++} and Zn^{++}) reactivate the enzyme at various degrees. The optimum concentration for these is : very weak for Zn^{++} and Fe^{++} (1.10^{-7}M), stronger for Mn^{++} (1.10^{-3}M) and Ca^{++} (1.10^{-2}M), while, for Mg^{++}, there is a threshold of activity with its greatest efficacy above 1.10^{-2}M.

3. Preliminary incubation with the aminoacid is indispensable; if this is neglected, only Mg^{++} and, to a lesser degree, Mn^{++} can be active agents, though to much smaller extent than after incubation, at p_H = 8,8 and at 37° C, of the enzyme in presence of alanine. Probably this is partly due to the reversion of the denaturation of the phosphatase in presence of the aminoacid, rather than to the slowness of the latter's reaction with the enzyme. Incubation with metallic ions, whether before or after the addition of alanine, is not really effective. The whole process, therefore, takes place as if the metal combined immediately with enzyme.

4. Preliminary incubation of purified phosphatase in presence of fluorides, orthophosphates and alkaline pyrophosphates, prevents reactivation by aminoacids and divalent cathions. Conversely, preliminary incubation of the enzyme in the presence of alanine strongly interferes with the inhibiting action of fluorides, orthophospates and alkaline pyrophosphates. We may deduce from this that aminoacids and the anions of the last-named salts combine with the same structural element in the active group of the phosphatases.

5. It is emphatically pointed out that the action of these agents, particularly that of cathions and of those forming complexes, on alkaline phosphatase can, as a rule, be effective only on purified products, and in certain definite metal-concentrations which vary with each of them. In the opposite case this action is a function of the presence of the metal and of substances forming natural complexes in the enzyme preparations. The frequent contradictions on this question found in the literature must be attributed to this fact; different, easily reproduceable results may, indeed, be obtained according to the method followed.

6. The importance of these facts is discussed regarding the constitution of alkaline phosphatase. The interchangeability of active metals does not argue in favour

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of the hypothesis that there exist, in various organs, phosphatases, each of which specifically contains some metal peculiar to the tissue in which it is found. Magnesium or, to a lesser degree, zinc, appears to be the most common mineral constituent of alkaline phosphatases, and appears to function as a structural element rather than as a catalytically active enzyme.

ZUSAMMENFASSUNG

1. Die gereinigte alkalinische Phosphatase (Phosphomonoesterase) wurde vollkommen inaktiviert durch fortgesetzte Dialyse gegen bidestilliertes Wasser und reaktiviert. Das erzielte hydrolytische Vermögen ist grösser als dasjenige des Initialproduktes, da das Enzym bis zu 700 millimolekülen Natrium β-Glyzerophosphat pro Minut und pro Gramm Stickstoff bei $p_H = 8,8$ und bei 37° spaltet ($Q_p = 170.000$).

2. Die maximale Reaktivierung der Phosphatase wurde nach Inkubation (2 Stunden) des Enzyms mit Alanin (1.10^{-2}M) bei $p_H = 8,8$ und durch Zusatz von Magnesiumionen verwirklicht. Unter gleichen Bedingungen wird das Enzym durch zweiwertige Kationen, wie Ca^{++}, Fe^{++}, Mn^{++} et Zn^{++}, in verschiedenen Graden reaktiviert. Es besteht für dieselben eine optimale Konzentration, welche für Zn^{++} und Fe^{++} schwach (1.10^{-7}M), und für Mn^{++} (1.10^{-3}M) und Ca^{++} (1.10^{-2}M) höher ist, während für Mg^{++}, das seine grösste Aktivität bei Konzentrationsgraden über 1.10^{-2} M erreicht, eine Wirkungsschwelle beobachtet wird.

3. Die Vorinkubation der Aminosäure ist erforderlich. Bei Unterlassung derselben werden allein Mg^{++} und Mn^{++}, wenn auch in einem geringeren Grade, zu Aktivatoren, aber ihre Wirksamkeit ist viel kleiner als nach Verbleiben des mit Alanin bereicherten Enzymes bei $p_H = 8,8$ und bei 37°. Alles vollzieht sich also wie wenn die Verbindung des Metalls mit dem Enzyme augenblicklich stattfinden würde.

4. Die Vorinkubation der gereinigten Phosphatase in Gegenwart von Fluoriden (Fluorüren), Orthophosphaten, alkalinischen Pyrophosphaten verhindert die Reaktivierung durch die Aminosäuren und die zweiwertigen Kationen. Umgekehrt, wird die hemmende Wirkung der Fluoriden (Fluorüren), der Orthophosphaten und der alkalinischen Pyrophosphaten durch die Vorinkubation des Enzymes in Gegenwart van Alinin stark gehindert. Hieraus kann man schliessen, dass die Aminosäuren und die Anionen dieser letzteren Salzen sich wahrscheinlisch mit dem gleichen Strukturelement der aktiven Phosphatasegruppe verbinden.

5. Es ist notwendig folgende Tatsache ganz besonders hervorzuheben : die effektorischen Agenten, insbesondere die Kationen und die Komplexbildner wirken nur regelmässig auf gereinigten Produkten und bei genau bestimmten Metallkonzentrationen, wobei letzteren für die einzelnen Metalle verschieden sind. Im entgegengesetzten Falle hängt diese Wirkung von dem Vorhandensein von Metallen und von natürlichen Komplexbildnern in den enzymatischen Präparaten ab. Die mannigfaltigen Widersprüche, die man in zahlreichen Arbeiten feststellt, müssen auf diese Ursache zurückgeführt werden. Verschiedene, leicht reproduzierbare Resultate können tatsächlich mit der angewandten Übungsmethode erzielt werden.

6. Die Bedeutung dieser Tatsachen bezüglich der Zusammensetzung der alkalinischen Phosphatase wird besprochen. Die Auswechselbarkeit der aktiven Metalle ist nicht günstig für die Annahme, dass Phosphatasen in den verschiedenen Geweben vorhanden sind, und dass jede Phosphatase ein Metall spezifisch enthält, welches dem Gewebe eigen ist, worin sie vorhanden ist. Das Magnesium, und, in einem kleineren Grade, das Zink, scheint der häufigste mineralische Bestandteil der alkalinischen Phosphatasen zu sein und die Rolle eines Strukturelementen eher als die eines katalytisch aktiven Koenzymes darin zu spielen.

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Bonedust-Meat Mixture as a Test Meal for Continuous Gastric Secretion.

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There is a limited choice of stimulation procedures of the gastric glands for physiological experiment. That probably most frequently used is stimulation by *histamine*. This substance seems to act directly on the parietal cells. Histamine therefore stimulates the glands in a Heidenhain pouch or in a totally denervated (transplanted) pouch. True physiological stimulation is more complex. The cephalic phase, which is a stimulation via vagus has been imitated by *direct electrical stimulation of vagus* or by *insulin*. In both cases the secretion is richer in enzymes than that induced by histamine.

It has been postulated that vagus stimulation liberates the pyloric hormone gastrin. This humoral substance acts on the secreting cells (possibly by liberating histamine). However, this hypothesis does not gain support from the fact that a Heidenhain pouch (which is vagally denervated) does not respond to vagal stimulation. If gastrin were really released and transported by the blood stream, why should it not have an effect on the pouch? The experiments by UVNÄS (1942) gave an explanation to that question. He found that the pyloric gastrin alone was not able to induce an acid secretion, but a cooperation with vagus stimulation directly on the secreting glands was necessary. Thus only those glands that were innervated by an intact vagus could respond to the liberated gastrin. BABKIN, SCHACHTER and NISSE

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(1944) objected to this view. Subsequent studies in many laboratories show that the gastrin mechanism is not yet understood.

Food stimulation offers a third stimulating mechanism (the gastric phase). This stimulation — the most physiological one — is unknown as to its intermediate action, but both the main stomach, the innervated and the denervated pouch respond to it.

It has become important to choose the correct stimulation method in the numerous experiments in which inhibiting substances are tested. If the stimulation chain for the cephalic phase schematically is written as *vagus* → *ganglion* → *end neuron* → *nerve ending* → *acetylcholine* → *gastrin* → *histamine* → *secretion*, it is easy to understand that any substance that interrupts this chain may also stop the secretion. For instance many quarternary ammonium derivatives that block the synaptic transmission or have anticholinergic effects inhibit the secretion after insulin, but the stimulation by histamine is unaffected. As a matter of fact only few substances are found to inhibit the histamine secretion (histaminase, antihistamine, entero(uro)-gastrone, mucus extracts, some pyrogenic substances and muscular exercise). Many more substances have been found to inhibit secretion otherwise induced. The different quarternary ammonium bases have become important in the last few years with the consequence that a suitable stimulation technique has been more and more in demand. Insulin is no doubt a good stimulation method in unnarcotized animals and man, and has been used to a great extent but in the case of laboratory animals that necessitates a fully innervated stomach preparation. Due to the easier technique for obtaining Heidenhain pouches a stimulation method suitable for such pouches was wanted.

A natural way was to try food, but not as ordinary meals as this gives secretion rates, that are inconstant and uncontrollable, but as a continuous administration of small amounts of meat. It should, in this connection, be pointed out that food stimulation is not equivalent to vagus stimulation. In another paper it will be shown that food has a shorter stimulation pathway.

Stimulation procedure.

Heidenhain pouch dogs were given balls of forcemeat as poor in fat as possible. For all experiments the same forcemeat was used (deep frozen). The forcemeat was either given alone, or with the addition to it of substances to be tested. The procedure was quite simple. Dogs

BONEDUST-

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fasted at least overnight, were fed every ten minutes with the food. Water was given ad libitum. The gastric juice was collected in a special measuring cylinder and analyzed for acidity and chloride (cf. ÖBRINK 1948).

Results.

Stimulation by forcemeat:

By giving the meat every ten minutes the secretion rate rose to a constant value, which was obtained within 1½—2 hours and could be maintained fairly constant for long periods. By varying the size of the meatballs the constant rate of secretion could be varied a little. It was found that the maximal secretion was very slight in comparison with the maximal capacity after histamine (1 : 10). Compare figs. 1 and 2.

Stimulation by bonedust-forcemeat:

It was necessary to find a better stimulating agent than forcemeat. As the dogs like forcemeat very much it was, however, thought wise to use forcemeat as a vehicle for some more specific secretagogue.

Many different things were tried without success. Different kinds of beef cubes and other meat extracts were all insufficient but on trying bonedust a good effect was obtained.[1]

The routine was to give the dogs forcemeat pieces of 10 grams into which the bonedust was added. By adding different amounts of bonedust different constant secretion rates could be obtained which is shown in fig. 1. The maximal secretion rate by this method was about 4—5 times higher than with meat alone. The relation to the maximal capacity after histamine was 1 : 2.3. The response to histamine in the same dog is given for comparison — fig. 2. It is obvious that a graded response can be obtained by this stimulation technique. The explanation will be discussed below.

It was of great interest to try to find out why bonedust was such an excellent substance for gastric stimulation. It is a powder from bone, which has been treated with benzene and then heated under increased water vapor pressure. It is practically free from fat and carbohydrates but contains 9 % protein and protein derivatives and small amounts of keratin and fluorine. Most of

[1] The idea to use bone-dust was of course developed from the knowledge that bone is a powerful stimulating agent. Bonedust is free from fat, and thus unable to liberate enterogastrone.

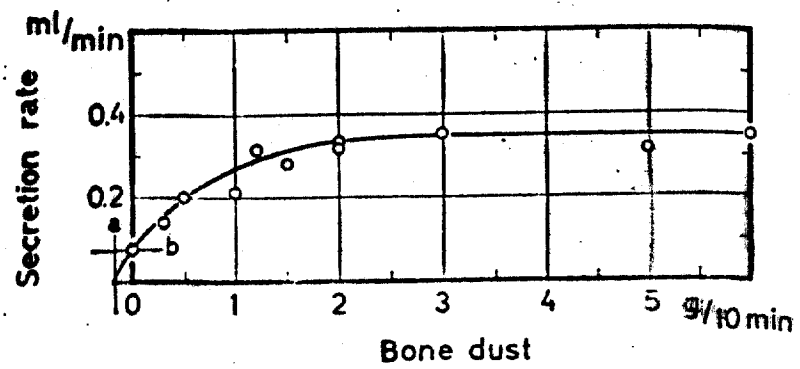


Fig. 1. *The relation between the amount of bonedust given to the dog every 10 minutes and the secretion rate.* When no bonedust was added the secretion was entirely due to the forcemeat (10 g). This rate (indicated by *b*) was actually the highest rate obtainable with forcemeat alone. The heavy line is obtained according to formula $v = v_{max}(1 - e^{-kr})$, where v = secretion rate, r = g bonedust, v_{max} and k = constants (cf. ÖBRINK 1948, p. 31). The vertical line *a* indicates the bonedust equivalent of 10 g forcemeat as estimated from its content of phosphorus. These two lines intersect the abscissa almost at the same point which indicates that the secretagogue effect of forcemeat may be due to its content of phosphate.

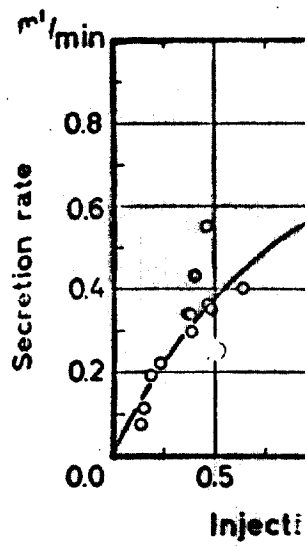
it consists of Ca-phosphate and carbonate. In the bone there is mostly Ca-*tri*phosphate and it seems probable that in the bonedust most of the 15.5 % of the phosphorus is forming *tri*-phosphate, which thus makes 77 % of the whole material.

It was therefore a natural consequence to substitute calcium-triphosphate for the bonedust in equal molar concentrations. Fig. 3 A shows the result indicating that most of the effect of bonedust apparently was due to its contents of calcium phosphate.

The next question to answer was: Is the Ca^{++}, the PO_4^{---} ion or their combination responsible for the effect? To answer this question *Ca-lactate* and *Na-triphosphate* were tried with the results shown in figs 3 B and 3 C. None of these substances had the effect of calcium triphosphate. As these two salts are highly ionized in comparison with the calcium phosphates two other salts were tried: *Ca-citrate* and *Na-glycerophosphate*. The first is a complex compound and in the latter the phosphate group is attached to the glycerine molecule.

The results — figs. 3 D and 3 E — show that the Ca-salt had no effect while the glycerophosphate had a marked effect.

The same results were obtained in another dog and there the effect of the glycerophosphate was even more obvious and quite similar to that of the Ca-phosphate and bonedust.



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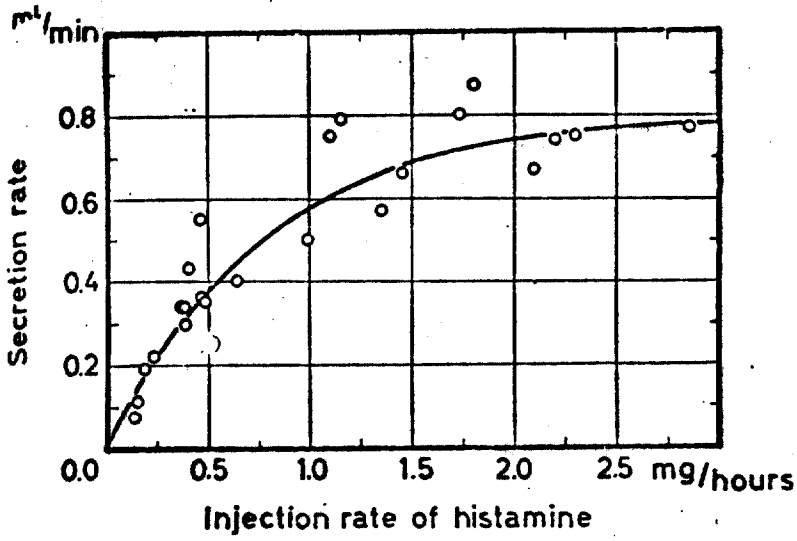

Fig. 2. The relation between constant intravenous histamine injection and secretion rate. From the same dog as in fig. 1.

Discussion.

It was found that the rather slight stimulating effect on the gastric glands evoked by forcemeat could be markedly increased by the addition of bonedust. The bonedust acted through its content of calcium phosphate and a graded dose-response curve appeared when the concentration of bone-dust was increased in the meat-bonedust mixture.

The explanation for the constancy of the secretion rate and the fact that this rate so nicely depended on the concentration of bonedust seems to be that a definite concentration gradient of the active substance between the gastric content and some part of the mucosa is the determining factor. It may therefore be easy to understand why the secretion could go on for at least one hour after withdrawing of the food and still on the same secretion level.

It was found that calcium phosphate could substitute the bonedust and that the Ca-ion both as the lactate and citrate was ineffective, while the phosphate ion in the rather stable combination as glycerophosphate had a similar activity as calcium phosphate. Sodium phosphate on the other hand was ineffective. No conclusive explanation can be given for the different secretagogue

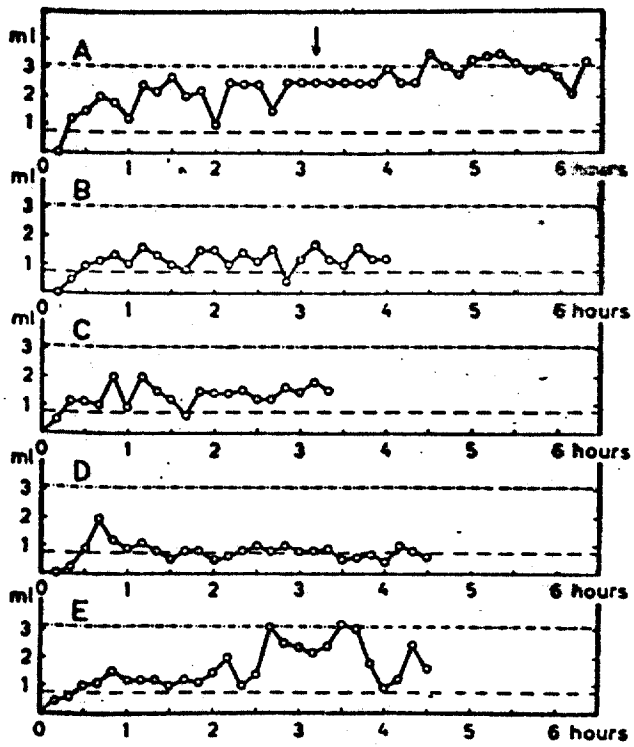

Fig. 3. *The secretion rate after 10 g of forcemeat every 10 minutes mixed with equimolar concentrations of different substances.* The lower horizontal line (— — — —) indicates the maximal secretion rate after 10 g forcemeat alone and the upper line (—·—·—) after 10 g forcemeat + 1.5 g bonedust.

A. $Ca_3(PO_4)_2$. At the arrow $CaCO_3$ was added to a molar concentration half of the phosphate.
B. Ca-lactate.
C. Na_3PO_4.
D. Ca-citrate.
E. Na-glycerophosphate.

properties of the phosphate salts. One factor that may be considered is the different charges of the ions. In the well buffered forcemeat the phosphate may appear as HPO_4^{--} and to a lesser degree as PO_4^{---}. These ions may have greater difficulties to permeate the gastric mucosa than has the glycerophosphate or the $Ca_3(PO_4)_2$. These suggestions will be tried out in planned experiments.

With the possible idea that phosphate was the responsible factor in bonedust, the stimulating effects of forcemeat and bonedust were compared with their contents of phosphorus. 10 g of

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Uvnäs, B., Ibidem 1942.

forcemeat contained phosphorus equivalent to 0.15 g bonedust and its stimulating effect equalled that of 0.16 g bonedust (cf. fig. 1). Thus it is possible that the entire stimulating effect of meat on the gastric glands is due to its content of phosphates.

No statement concerning the mode of action of the bonedust can, however, be given with certainty but it is demonstrated that bonedust is a very useful stimulating agent for the gastric secretion.

Summary.

An addition of bonedust to forcemeat increases the secretagogue effect on the gastric secretion. The bonedust exerts this powerful action mainly through its content of calcium phosphate.

It was shown that the phosphate ion was perhaps responsible for the effect.

By varying the concentration of bonedust in the meat-bonedust mixture and by giving it to dogs in small portions every 10 minutes it was possible to get constant and desired secretion rates over a period of many hours.

The expences of this investigation have been defrayed by grants from "August and Therése Anderssons Minne".

My thanks are also due to Mrs Maj Ulfendahl for valuable assistance.

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688

Dott. SIGISMONDO OSSER

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DOSE MINIMA LETALE LONTANA E CAUSA DELLA MORTE IN SEGUITO AD INIEZIONI ENDOVENOSE DI FOSFATO BISODICO

I) RICERCHE PRECEDENTI.

Il primo a saggiare l'azione dei sali di fosforo sugli organismi degli animali fu FLACK (1872) (1). Egli osservò nei cani scialorrea e contrazioni crampiformi agli arti posteriori. Non giunse però a nessuna conclusione sulla tossicità di questi composti. Parimenti non dice il modo con cui li somministrava.

Successivamente GAMGEL, PRIESTLY e LARMUTH, studiando nel 1877 l'azione farmacologica del fosfato bisodico, notarono la costante comparsa della tetania nei conigli dopo l'introduzione endovenosa di 0,2 gr. di fosforo per Kg. di animale. KOBERT, un anno dopo (3), osservò nei cani, per azione del fosfato bisodico, ipertermia ed aumento della pressione sanguigna. Saggiò anche gli altri fosfati di sodio, ma non attribuì a tutti questi composti, fosfato bisodico compreso, alcuna importanza tossica.

Invece MÜNTZER nel 1898 confermò la tossicità di questo sale, non solo, ma ne fissò anche, per primo, la dose minima mortale, che, secondo lui, è uguale a gr. 4 per 1 Kg. di animale. Questo autore iniettava endovena, ma non dice nè il titolo della soluzione, nè la velocità di iniezione usate.

Anche KUNKEL (4), tre anni dopo (1901), ricercò la dose minima letale del fosfato bisodico, e la dichiarò uguale a gr. 5,4 per Kg. di animale (coniglio).

Le ricerche più complete sul fosfato bisodico si devono a SABBATANI (1904), che iniettava nella giugulare di cani e di conigli una soluzione al 17 % del farmaco (5). Veramente lo scopo principale delle sue ricerche fu di studiare l'azione immobilizzante sul calcio dei vari fosfati. Ma egli stabilì anche la dose minima mortale del fosfato bisodico per i conigli, che, secondo lui, doveva essere uguale a gr. 2,1 per Kg. di animale. Però egli iniettava velocemente e generalmente non seguiva gli animali oltre il secondo od il terzo giorno. SABBATANI osservò la costanza della comparsa di tetano (a dosi sufficienti del farmaco), inoltre nei carnivori (cani) diuresi ed ipertemia.

Secondo SABBATANI il fosforo diminuisce la concentrazione ionica dei sali di calcio nel sangue e nei tessuti. Se l'animale, dice l'Autore, non muore durante i primi attacchi tetanici, la decalcificazione grave diminuisce per la presenza di acido carbonico e per la trasformazione del fosfato bisodico in fosfato monosodico, che ha poca azione decalcificante, e l'animale si rimette.

Successivamente il fosfato monosodico (che è di reazione acida al tornasole) produce l'intossicazione acida di vari organi. SABBATANI vide, per esempio, la degenerazione grassa nel fegato dei cani. Anche Mc CALLUM e VOEGTLIN (1909) osservarono l'antagonismo tra calcio e fosforo nella tetania.

Invece GREENWALD (6), passando in rassegna, nel 1915, i lavori più importanti sugli ortofosfati e riportando anche delle osservazioni personali (il lavoro del SABBATANI non è però citato da lui) dice che i fosfati di sodio non hanno alcuna azione farmacologica ed afferma che i fenomeni tossici osservati dai varî autori sono dovuti piuttosto alle impurità contenute nei composti da loro adoperati.

BINGER nel 1917 (che però non cita e forse non conosce le esperienze di SABBATANI) riafferma che la tossicità dei fosfati è dovuta all'azione specifica dell'jone fosforico alla quale si aggiunge la reazione del mezzo in cui si trova (7). Afferma inoltre che la diminuzione del calcio nel sangue è una condizione necessaria per la comparsa della tetania, ma non è, però, una condizione sufficiente. Egli dice che i sintomi tetanici compaiono quando la quantità di calcio nel siero si abbassa fino a 6 mmg. per 100 cc. e chiama que-

sto abbassamento il « livello tetanico ». Per ridurre la quantità di calcio a 6 mmg. per 100 cc. di siero occorrono, secondo BINGER, 0,2 gr. di ione fosforo (come GAMGEE - v. sopra). Nota infine, però, che il tetano può comparire con 0,3 gr. di fosforo anche senza che la quantità di calcio sul siero si abbassi necessariamente a 6 mmg. per 100 cc., e ciò quando le soluzioni sono acide. Ricorderò ancora le ricerche di HENDERSON (8) e di SÖRENSEN (9), secondo i quali il fosforo inorganico si trova nell'organismo sotto forma appunto di sali di sodio; la funzione fisiologica di questi sali consiste nel mantenere la neutralità del sangue avendo essi la capacità di legare gli acidi e le basi. Menzionerò infine STARKENSTEIN (10) (1911), che ha visto che la tetania prodotta da fosfati di sodio poteva essere prevenuta con delle iniezioni di cloruro di calcio.

* * *

Da questi brevi cenni si vede subito che l'azione dei fosfati di sodio, e del fosfato bisodico in particolare, non fu mai oggetto di uno studio sistematico e severo, nè fu mai condotto con un metodo unico. Infatti rileviamo anzitutto che non esiste accordo, nemmeno approssimativo, sulla dose minima mortale di questo farmaco. E mentre per MÜNTZEN essa è uguale a circa 4 gr. per Kg. di animale, per KUNKEL è di gr. 5,4 e per SABBATANI è pari a gr. 2,1 appena.

Siccome molti autori non indicano nè la via di somministrazione (la quale può essere endovenosa, sottocutanea e gastrica), nè dicono se determinavano la tossicità lontana o la tossicità immediata, possiamo pensare che la diversità dei loro risultati dipende appunto dal fatto che essi non seguivano metodi di indagine comparabili. Gli effetti del farmaco dipendono invece, non solo dalla via di somministrazione, ma anche dalla velocità di essa e dalla grandezza della dose, che può uccidere gli animali subito dopo l'esperimento oppure dopo un certo periodo di tempo da questo, come vedremo in seguito. Notiamo poi che le alterazioni anatomo-patologiche prodotte dal fosfato bisodico non furono affatto studiate dalla maggior parte degli autori. Solo SABBATANI parla della degenerazione grassa del fegato dei cani.

La comparsa del tetano fu invece oggetto delle osservazioni più costanti e notiamo anche una certa unità di vedute sul meccanismo di produzione di esso, specialmente per merito di SABBATANI e di BINGER. Ed esiste anche accordo sulla quantità minima di fosforo (0,2 gr. per un chilo di animale) somministrato sotto forma di fosfato bisodico, capace di far comparire i primi accessi tetanici, ossia il cosidetto « livello tetanico » (GAMGEL, BINGER).

Dall'altra parte abbiamo delle poco spiegabili conclusioni di GREENWALD e di KOBERT, che negarono addirittura qualsiasi azione farmacologica ai fosfati di sodio in genere ed al fosfato bisodico in particolare. Riesce particolarmente incomprensibile l'affermazione di GREENWALD, che attribuisce la sintomatologia di intossicazione prodotta dai fosfati, osservata da lui e dai suoi predecessori, esclusivamente alle impurità contenute nei suddetti sali.

* * *

Lo scopo delle mie ricerche è, appunto, quello di colmare alcune di queste lacune sull'azione del fosfato bisodico. Determinerò anzitutto la dose minima mortale. Osserverò parallelamente la sintomatologia generale provocata dalla somministrazione di questo farmaco e finalmente descriverò alcune alterazioni anatomo-patologiche dei principali organi (fegato, rene, cuore, polmoni) dovute alla sua azione.

II) ESPERIENZE.

Animali da esperimento furono conigli, che sceglievo apparentemente sani, digiuni da circa 24 ore. Ho adottato il metodo delle iniezioni endovenose in una vena giugulare che, previa fissazione dell'animale sull'apposito apparecchio, mettevo allo scoperto introducendovi una cannula unita, per mezzo di un tubo di gomma, con una buretta graduata. Finita l'iniezione, dopo un'accurata emostasi, suturavo la ferita con un filo di seta. Gli animali in osservazione furono nutriti da me personalmente a orario fisso.

Adoperavo una soluzione 3/4 normale di fosfato bisodico, contenente gr. 89,56 di farmaco in 1000 cc. di acqua. La preparavo fresca ogni due-tre giorni.

Cercavo la *dose minima letale lontana*, cioè la quantità minima

del farmaco che, iniettata nelle vene, lo uccide, non durante l'iniezione, ma qualche tempo dopo questa. Nell'iniezione si procede molto lentamente in modo da non compromettere la vita dell'animale, sospendendo eventualmente per qualche tempo, non appena incominci a dar segni acuti di forte malessere, per riprenderla solo quando questi segni sono scomparsi.

Terminata l'iniezione si pone l'animale in osservazione. Dopo la morte si procede all'autopsia ed all'esame sistematico macro e microscopico dei vari organi per vederne le alterazioni ed il meccanismo della morte. Saggiando così con quantità diverse di un dato farmaco un certo numero di animali, noi arriviamo a determinare con esattezza la dose minima con la quale si provoca la morte di essi.

Passo ora alla descrizione degli esperimenti da me eseguiti e poi riassumerò le osservazioni fatte nel corso di questi.

I^a ESPERIENZA — 27 - XI - 1931.

Ad un coniglio di 1600 gr. inietto nella v. giugulare d. cc. 40 per Kg. di soluz. 3/4 N di fosfato bisodico, complessivamente quindi 67 cc. di soluzione. Durata dell'iniezione 96'.

Dopo l'iniezione di 24 cc. di soluz. l'animale presenta un accesso di contrazioni tonico-cloniche degli arti e del dorso. Questi accessi si fanno sempre più frequenti col progredire dell'iniezione. Verso la fine dell'esperimento l'animale resta immobile, il respiro è lento e stertoroso. Muore appena slegato. Il cuore batte dopo che è cessato il respiro.

Questo coniglio ricevette complessivamente gr. 5,731 e gr. 3,582 di sostanza per Kg. Le prime contrazioni comparvero dopo l'iniezione di 16 cc. di soluzione per Kg.

Autopsia (fatta subito dopo la morte) rivela:
Cuore: flaccido, in diastole, pieno di sangue liquido, scuro.
Polmoni: di aspetto normale.
Fegato: di aspetto normale.
Rene: di aspetto normale.
Reperto microscopico: non si rilevano alterazioni degne di nota.

II^a ESPERIENZA — 27 - XI - 1931.

Inietto nella vena giugulare d. di un coniglio di 1150 gr., 30 cc. di soluz. 3/4 normale di fosfato per Kg.: complessivamente quindi 34 ½ cc. di soluzione. Durata dell'iniezione 35'. Dopo la somministrazione di 20 cc. di soluzione compaiono i primi accessi di contrazioni tonico-cloniche agli arti e al dorso. Respiro stertoroso e frequente. Questi accessi si fanno sempre

più frequenti col progredire dell'iniezione. Questo animale ricevette complessivamente gr. 3,045 di sostanza e gr. 2,686 per Kg. Le prime contrazioni comparvero dopo l'iniezione di 17,35 cc. di soluzione per Kg.

Liberato il coniglio, resta con arti rigidi ed estesi, la nuca estesa. Respiro stertoroso e profondo. Dopo 10 minuti riesce a rimettersi prono per qualche minuto, poi ricade e muore dopo 80' in preda a tremori e convulsioni generalizzate. La ferita suturata sanguina fino alla morte. Il cuore batte dopo cessato il respiro.

Autopsia (fatta dopo 12 ore).

Cuore: in sistole, un po' pallido, pieno di coaguli neri.

Polmoni: emorragie diffuse lentiformi.

Fegato: lieve edema.

Rene: lieve edema, corticale ingrossata.

All'esame microscopico si osservano nel rene: irrorazione normale: nella corticale gravi alterazioni dei tubuli consistenti in zone numerose di necrosi da coagulazione e zone di rigonfiamento; a carico degli epiteli delle anse di Henle si trovano dei fatti distruttivi del protoplasma, che talora è conservato soltanto alla base dell'epitelio ed attorno ai nuclei; gli epiteli dei tubuli collettori sono così rigonfi da occludere completamente il lume tubulare.

Miocardio: lievi fatti di edema.

Polmoni: tutti gli alveoli sono ripieni di essudato fibrinoso e di leucociti.

Fegato: alcune cellule epatiche hanno nel loro protoplasma dei piccoli vacuoli in numero di 2-3 per cellula.

III^a ESPERIENZA — 29 - II - 1933.

Inietto nella v. giugulare d. di un coniglio di 1100 gr., 28 cc. di soluzione 3/4 normale di fosfato bisodico per Kg., complessivamente quindi cc. 30,8. Durata dell'iniezione 79'. Dopo la somministrazione di 16 cc. di soluzione compaiono i primi accessi di contrazioni tonico-cloniche agli arti ed al tronco, che si fanno più frequenti col progredire dell'iniezione. Il polso che, colla comparsa delle prime contrazioni, si era fatto frequente ed irregolare, ridiventò verso la fine di nuovo regolare. Questo coniglio ricevette complessivamente gr. 2,758 di sostanza, ossia gr. 2,507 per Kg. Le contrazioni comparvero dopo la somministrazione di cc. 17,5 di soluzione per Kg.

Liberato l'animale, presenta contrazioni tonico-cloniche degli arti, il dorso e la nuca si portano spesso in estensione. Muore in questo stato dopo 1 ora e 40'. La ferita suturata sanguina fino alla morte. Il cuore batte dopo cessato il respiro.

Autopsia (fatta subito dopo la morte).

Cuore: in sistole, un po' pallido.

Polmoni: emorragie diffuse e lentiformi.

Fegato: edema lieve.

Rene: edema lieve. La corticale è ingrossata.

Esame microscopico - Rene: l'irrorazione appare normale; nella sostanza corticale si notano gravi alterazioni dei tubuli, consistenti in zone numerose di necrosi da coagulazione e zone di rigonfiamento; a carico degli epiteli delle anse di Henle si rilevano dei fatti distruttivi del protoplasma, che talora è conservato soltanto alla base dell'epitelio ed attorno ai nuclei; gli epiteli dei tubuli contorti sono rigonfi tanto da occludere completamente il lume tubulare.

Miocardio: fatti di edema lieve.

Polmoni: tutti gli alveoli sono ripieni di essudato fibrinoso e di eritrociti.

Fegato: alcune cellule epatiche hanno nel loro protoplasma dei piccoli vacuoli in numero di 2 o 3 per ogni cellula.

IV^a ESPERIENZA — 29 - II - 1933.

Inietto nella v. giugulare d. di un coniglio di 1000 gr., 26 cc. di soluzione 3 4 normale di fosfato bisodico. Durata dell'iniezione 26'. Dopo la somministrazione di 17 cc. di soluzione compaiono le contrazioni tonico-cloniche agli arti ed al tronco, mentre il polso si fa frequente ed irregolare. Verso la fine dell'esperimento il polso ritorna ad essere regolare, ma gli accessi convulsivi si fanno più gravi.

Quando è liberato continuano le contrazioni tonico-cloniche generalizzate, la nuca si porta spesso in estensione. Muore in questo stato dopo 70'. La ferita suturata sanguina fino alla morte.

Autopsia e reperto istologico uguali a quelli dell'esperienza III.

Questo animale ricevette gr. 2.327 di sostanza per Kg.

V^a ESPERIENZA — 1 Marzo 1933.

Inietto nella v. giugulare del coniglio di 1300 gr., 24 cc. di soluzione 3/4 normale di soluzione di fosfato bisodico per Kg., complessivamente quindi 31,2 cc. Durata dell'iniezione 40'. Dopo la somministrazione di 18 cc. di soluzione compaiono i primi accessi delle contrazioni tonico-cloniche agli arti accompagnati dall'estensione del tronco e della nuca. Il respiro diventa frequente e stentato. Scialorrea. Questi fenomeni vanno aggravandosi verso la fine dell'esperimento.

Questo animale ricevette complessivamente gr. 2,794 di sostanza, ossia gr. 2,149 di sostanza per Kg. Le prime contrazioni comparvero dopo la somministrazione di gr. 13.85 cc. di soluzione per Kg. Liberato, l'animale conserva l'atteggiamento che aveva nell'apparecchio di contezione. Poi a di

stanza di circa un quarto d'ora l'uno dall'altro ha tre classici attacchi tetaniformi. Questi dopo si fanno più frequenti.

La ferita suturata sanguina per circa 3 ore.

Dopo 5 ore lo stato tetanico persiste. Accostando la mano si avverte un tremore continuo dei muscoli del dorso. Contrazioni fibrillari periorali. Respiro profondo; persiste la scialorrea.

Dopo 8 ore le contrazioni si fanno più rare. Dopo qualche tentativo riesce a sollevarsi sugli arti posteriori. E' scomparsa la rigidità del dorso. Respiro 52, polso 84.

Dopo 18 ore. Ritorna lo stato tetanico. Respiro 40, polso 66. Corpo freddo.

Dopo 23 ore. Come sopra. Geme.

Dopo 24 ore. Muore in stato tetanico. Il cuore batte dopo cessato il respiro.

Autopsia (fatta subito dopo la morte). Cuore in diastole, di aspetto normale, pieno di sangue, molto scuro, liquido.

Polmoni: rosei, lievemente edematosi, con emorragie petecchiali e puntiformi.

Fegato: lievemente edematoso.

Rene: edema lieve, un po' pallido. La sostanza corticale appare ingrossata.

Reperto microscopico:

Rene: glomeruli compresi, di forma irregolare; lo spazio intracapsulare scomparso; gravi alterazioni a carico dei tubuli contorti che, in certe zone, sono sostituiti da un tessuto quasi omogeneo nel quale a mala pena si distinguono i limiti fra i resti dei tubuli; il lume di essi è scomparso, come pure sono scomparsi i nuclei, di cui si vedono solo qua e là dei resti. In questa massa spiccano i glomeruli sopratutto per la loro più intensa colorazione. Il protoplasma degli epiteli dei tubuli retti è alterato e parzialmente sfaldato.

Polmoni: alcuni alveoli sono infarciti di sangue.

Fegato: piccoli vacuoli in cellule epatiche, in numero di 2-3 per ciascuna.

VI° ESPERIENZA — 2 Marzo 1933.

Inietto nella v. giugulare d. di un coniglio, di 1370 gr., 22 cc. di soluzione 3/4 normale di fosfato bisodico per Kg., complessivamente quindi cc. 30,14. Durata dell'iniezione 45'. Dopo la somministrazione di 25 cc. di soluzione compaiono i primi accessi di convulsioni tonico-cloniche agli arti, che verso la fine si fanno più frequenti. Questo animale ricevette complessivamente 2,709 gr. di sostanza, ossia gr. 1,970 per Kg. Le prime contrazioni comparvero dopo la somministrazione di cc. 18 di soluzione per Kg. di animale.

Liberato, il coniglio assume quasi subito l'atteggiamento normale e sembra soltanto stordito. Dopo un'ora mangia. La ferita suturata sanguina per circa mezz'ora.

Muore dopo 3 giorni e sta apparentemente bene fino a 3 ore prima della morte. Allora compaiono segni di progressivo indebolimento, che si aggravano fino all'esito letale. Il cuore batte dopo cessato il respiro.

Autopsia e reperto microscopico uguali a quelli dell'esperienza precedente.

VII^a ESPERIENZA — 9 - IV - 1932.

Inietto, nella v. giugulare d. di un coniglio, di 1100 grammi, cc. 20 per chilo di soluzione 3/4 N di fosfato bisodico, complessivamente quindi cc. 22 di soluzione.

Durata dell'iniezione 30'. Dopo la somministrazione di 18 cc. di soluzione compaiono i primi accessi di contrazioni tonico-cloniche agli arti, accompagnati dall'estensione del dorso e della nuca. Questi accessi si fanno più frequenti verso la fine dell'iniezione.

Questo animale ricevette complessivamente gr. 1,970 di sostanza, ossia gr. 1, 791 di sostanza per chilo. Le prime contrazioni comparvero dopo l'iniezione di 16,4 cc. di soluzione per Kg.

Liberato, il coniglio assume quasi subito l'atteggiamento normale. E' un po' abbattuto e nel primo giorno mangia poco. La ferita suturata non sanguina.

Dopo 2 giorni: vivace, mangia bene. Peso 1040 gr.

Dopo 5 giorni: vivace, mangia bene. Peso 840 gr.

Dopo 7 giorni muore con segni di progressiva debolezza. Pesa 800 gr.

Questo animale perdette complessivamente circa il 27 % del peso primitivo.

Autopsia (fatta subito dopo la morte). Cachessia notevole.

Cuore: in sistole di aspetto normale.

Polmoni: rosei, un po' carichi. Non edema.

Intestino: notevolmente ridotto e contratto.

Fegato: notevolmente ridotto di volume; a superficie leggermente granulosa; di colore rosso-bruno molto scuro.

Rene: di volume normale, lievemente edematoso.

Esame istologico:

Rene: lieve iperemia dei glomeruli e dei capillari nella parte prossimale della corticale. Gli epiteli dei tubuli contorti sono in preda, in parte, a notevole rigonfiamento, con integrità del nucleo e scomparsa del lume canalicolare, ed, in parte, a degenerazione granulosa con distruzione della parte del protoplasma rivolta verso il lume, per cui in certi punti si vede soltanto un reticolo a larghe maglie nel quale si distingue ancora molto bene

il nucleo, che appare intatto. In alcune parti del preparato i tubuli appaiono sostituiti da una sostanza omogenea compatta, debolmente colorato dall'ematossilina, con sfumatura giallognola, senza nuclei, nè lume.

Queste alterazioni si fanno più lievi man mano che si procede verso la papilla renale e consistono in una degenerazione granulosa del protoplasma con sfaldamento delle parti di esso verso il lume.

Nelle zone ancora più distali gli epiteli sono integri e soltanto nel lume di alcuni tubuli si vedono dei cilindri omogenei di colorito grigio-giallastro.

Miocardio: atrofia delle fibre tanto che i nuclei sembrano ingranditi.

Polmoni: nell'interno degli alveoli si vede qualche epitelio desquamato e qualche eritrocita.

Fegato: Evidente rimpicciolimento delle cellule.

VIII^a ESPERIENZA — 14 - IX - 1932.

Inietto nella v. giugulare d. di un coniglio di 1400 gr., cc. 12 per chilo di soluzione 3/4 di fosfato bisodico, complessivamente quindi cc. 16,8 di soluzione.

Durata dell'iniezione 24'.

Nulla da rilevare durante l'esperimento. La ferita suturata non sanguina. Questo animale ricevette complessivamente gr. 1.505 di sostanza, ossia gr. 1,075 di sostanza per Kg.

Liberato, l'animale assume subito l'atteggiamento normale e dopo un'ora mangia.

Dopo un giorno sta bene, mangia.

Dopo 5 giorni vivace, mangia. Peso 1200 gr.

Dopo 11 giorni vivace, mangia. Peso 950 gr.

Dopo 12 giorni muore con segni di progressiva debolezza.

Questo animale perdette complessivamente circa 40 % del peso primitivo.

Autopsia (fatta subito dopo la morte), cachessia notevole.

Cuore: in sistole, di aspetto normale.

Polmoni: di colore roseo un po' carico.

Fegato: notevolmente impicciolito, ridotto ad una listerella applicata al diaframma, di colore rosso-nerastro, molto carico.

Rene: di volume normale, lievemente edematoso.

Esame microscopico:

Rene: sanguificazione dei glomeruli normale. Alcuni di essi sono compressi, lo spazio capsulare in questi è scomparso e sono a contorno irregolare. I capillari dei primi tratti dei tubuli contorti sono dilatati e pieni di sangue. La degenerazione dei tubuli contorti è meno estesa che nell'esperienza precedente ed è limitata quasi esclusivamente alla cortex-corticis, dove gli epiteli presentano talora una parziale distruzione e talora sono in

preda a degenerazione granulosa. Nel resto della corteccia si nota soltanto un maggiore ravvicinamento dei nuclei degli epiteli, che sono più stipati. Nel lume dei tubuli collettori si nota un reticolo debolmente colorato con ematossilina, che occupa soltanto una parte del lume.

Polmoni: in alcune zone i capillari sono dilatati e si trova qualche eritrocita negli alveoli.

Nel miocardio e nel fegato il volume delle cellule appare ridotto al punto che i nuclei sembrano ingranditi.

IX^a ESPERIENZA — 18 - IV - 1932.

Inietto nella v. giugulare di un coniglio di 1300 gr., 11 cc. di soluzione 3/4 N di fosfato bisodico per Kg., complessivamente quindi 14 cc. di soluzione.

Durata dell'esperimento 25'. Nulla da rilevare durante l'iniezione. La ferita suturata non sanguina.

Questo animale ricevette in tutto gr. 1280 di sostanza, ossia gr. 0,985 per Kg.

Liberato, il coniglio assume subito l'atteggiamento normale e mangia dopo pochi minuti.

Nei giorni successivi sta apparentemente bene e mangia voracemente. Dimagra però durante i primi 30 giorni fino a ridurre il proprio peso a 1010 gr. Poi riprende e dopo 40 giorni arriva di nuovo a 1250 gr.

X^a ESPERIENZA — 18 - IV - 1932.

Inietto nella v. giugulare d. di un coniglio di 1700 gr., 10 cc. di soluzione 3/4 normale di fosfato bisodico per Kg., complessivamente quindi 17 cc. di soluzione. Durata dell'esperimento 30'. Nulla da rilevare durante l'iniezione. La ferita suturata non sanguina.

Questo animale ricevette complessivamente gr. 1,521 di sostanza, ossia gr. 0,895 di sostanza per Kg.

Liberato, il coniglio non dà segni di sofferenza e mangia dopo pochi minuti.

Nei giorni successivi è vivace e mangia molto. Ma nei primi 25 giorni dimagra fino a ridurre il proprio peso a soli 1350 gr.

Poi riprende e dopo 35 giorni pesa 1550 gr.

XI^a ESPERIENZA — 16 - IV - 1932.

Inietto nella v. giugulare d. di un coniglio di 1350 gr., 8 cc. di soluzione 3/4 N di fosfato bisodico per Kg. Complessivamente quindi 10,8 cc. di soluzione.

Durata dell'esperimento 16'. Nulla da rilevare durante l'iniezione. La

ferita suturata non sanguina. Questo animale ricevette complessivamente 0,902 di sostanza, ossia gr. 0,716 di sostanza per Kg.

Liberato, il coniglio non presenta segni di sofferenza e mangia dopo pochi minuti.

Nei giorni successivi sta sempre bene, mangia molto, ma dimagra nei primi 30 giorni fino a ridurre il proprio peso a 1060 gr.

Dopo questo periodo incomincia a riprendere il peso.

TAVOLA RIASSUNTIVA DEGLI ESPERIMENTI

N. delle esperienze	Peso coniglio in gr.	Fosfato bisodico iniettato		Fosforo in gr. per kg.	Dose che provocò le prime contraz.		Esiti
		in gr.	in gr. per kg.		fosfato bis. in gr. per kg.	Fosforo in gr. per kg.	
1	1600	3,731	3,582	0,310	1,432	0,124	Muore subito dopo l'iniezione
2	1150	3,045	2,686	0,203	1,554	0,135	Morto dopo ore 1.20' dall'iniezione
3	1100	2,758	2,507	0,202	1,296	0,112	Morto dopo ore 1.40' dall'iniezione
4	1000	2,327	2,327	0,201	1,522	0,131	Morto dopo ore 0.40' dall'iniezione
5	1300	2,794	2,149	0,187	1,226	0,107	Morto dopo ore 24 dall'iniezione
6	1370	2,709	1,370	0,170	1,612	0,139	Morto dopo 3 giorni
7	1100	1,970	1,791	0,155	1,468	0,126	" " 7 "
8	1400	1,505	1,075	0,093	Non ebbero contrazioni		" " 12 "
9	1300	1,280	0,985	0,085	" "	"	Vive
10	1700	1,521	0,895	0,077	" "	"	"
11	1350	0.902	0,716	0,062	" "	"	"

Riassunto critico.

Cercherò ora di riassumere i risultati di queste serie di esperimenti:

1) Ho visto anzitutto che con dosi superiori a gr. 2,149 di fosfato bisodico per Kg., gli animali morivano o durante l'iniezione (esp. I), o brevissimo tempo dopo. Questo tempo variava da 40 a 100 minuti primi, ed i conigli soccombevano in preda a contrazioni tetaniche fortissime (esp. II, III, IV). Con le dosi inferiori a gr. 2,149 per Kg. gli animali morivano dopo un periodo di tempo molto più lungo ed in rapporto diretto con la quantità di farmaco usato. La morte di questi conigli non era accompagnata da comparsa di accessi tetanici o dalla loro ricomparsa, quando essi ebbero luogo durante l'esperimento (esp. V - VII).

La dose più piccola che provocò la morte del coniglio fu di

gr. 1,075 di farmaco per Kg. di animale. Il coniglio trattato con 0,985 gr. per Kg. sopravvisse, come pure sopravvissero costantemente i conigli con le dosi ancora più piccole (esp. IX - XI). Posso quindi affermare con certezza che la dose minima letale lontana del fosfato bisodico per i conigli, secondo le mie esperienze, è uguale a gr. 1,075 di questo sale per Kg. di animale, cioè è uguale a gr. 0,093 di fosforo.

Questa dose è notevolmente più piccola di quella segnata dagli altri autori. La discordanza è dovuta indubbiamente alla diversità di metodo. E' dovuta, cioè, al fatto che io iniettavo lentamente e non ho mai superato la velocità di 1 cc. di soluzione in 1 minuto per un Kg. di animale. Inoltre io ho tenuto gli animali in osservazione per un periodo di tempo assai lungo, fino a quando cioè essi non dimostravano di avviarsi verso un sicuro benessere.

2) Un'osservazione degna di rilievo fu la comparsa di contrazioni tetaniformi nei conigli dopo la somministrazione di una quantità relativamente costante di farmaco. Questa quantità oscillò tra un massimo di gr. 1,612 ed un minimo di gr. 1,296 di fosfato bisodico per Kg. (*). Come media per provocare queste contrazioni occorrono quindi gr. 1,444 di farmaco per Kg., uguale a gr. 0,125 di fosforo per Kg.

Questa è anche inferiore a quella segnata da Ganger e Bruger (0,2 gr. di fosforo per Kg.), evidentemente in rapporto colla velocità di iniezione.

3) Interessantissima fu la constatazione che i conigli morti dopo un periodo di tempo di qualche giorno dall'iniezione dimagravano notevolmente. Essi perdevano prima di morire dal 24 al 40 % (!) del peso primitivo. Anche quelli sopravvissuti dimagravano in varia misura. Ma dopo aver raggiunto in un certo numero di giorni il massimo del calo del peso, lo riacquistavano gradatamente, come risulta dalle cifre riportate nelle esperienze. Questo dimagramento contrastava con la apparente vivacità degli animali e con la loro voracità normale.

(*) Questa diversità può dipendere o da condizioni individuali degli animali o, in parte, forse, da piccole differenze nella velocità dell'iniezione.

4) Due volte ho constatato scialorrea (esp. III e V), che era già stata notata da Flack nel '872.

5) L'osservazione che le ferite suturate sanguinavano per un certo tempo dopo l'esperimento nei conigli trattati con dosi più alte di farmaco trova una facile spiegazione se si ricorda che l'anione fosforico è un reattivo del catione calcio. Così la coagulazione del sangue, per cui il calcio è indispensabile, fu impedita per un periodo di tempo tanto più lungo quanto più grande fu la quantità di fosforo iniettato.

6) In tutti gli animali morti la funzione respiratoria cessava prima della cardiaca.

7) Per quanto riguarda i reperti anatomo-patologici debbo dire che la trattazione completa di essi esorbiterebbe dai limiti di questo lavoro, tanto più che le osservazioni fatte in precedenza in questo campo sono quasi nulle. Mi sono limitato quindi a vedere le alterazioni del cuore, dei polmoni, del fegato e del rene. Le alterazioni di questi organi (escludo l'esperienza I, dove non si sono trovate alterazioni degne di nota essendo la morte intervenuta rapidamente) furono le seguenti. A carico del miocardio nulla di caratteristico nè all'esame macroscopico, nè a quello istologico. A carico dei polmoni nei conigli trattati con le quantità più grandi del farmaco, delle emorragie puntiformi e lentiformi; nei conigli trattati con quantità di farmaco più piccole i polmoni erano rosei, di aspetto pressochè normale; all'esame istologico trovai pochi eritrociti negli alveoli assieme ad una modica quantità di essudato in misura tanto più modesta quanto più piccola era la quantità di farmaco iniettata. Le alterazioni del fegato erano diverse a seconda della quantità di farmaco con cui erano stati trattati gli animali ed a seconda, quindi, del tempo di sopravvivenza. Negli animali morti dopo qualche giorno dall'iniezione il fegato era ridotto notevolmente di volume e più scuro del normale, mentre al microscopio non vidi che fatti di atrofia semplice. Negli animali morti in breve tempo invece osservai che macroscopicamente il fegato era lievemente edematoso ed al microscopio vidi nelle cellule epatiche dei vacuoli in numero di due o tre in ogni cellula.

Assai costanti furono le alterazioni dei reni. Macroscopicamente essi erano tutti un po' pallidi, con la corticale ingrossata, mentre

al microscopio erano palesi dei fatti di nefrosi tanto più lievi quanto più piccola era la quantità di farmaco iniettata.

8) Dal complesso delle mie osservazioni sintomatologiche ed anatomo-istopatologiche credo di poter affermare che nella morte da fosfato bisodico si debba distinguere quella immediata, dovuta alle alte dosi del farmaco, da quella lontana. Con le prime infatti la sintomatologia è dominata dagli accessi convulsivi gravi, tetano-simili, che compaiono costantemente non appena sia stata introdotta nell'organismo una determinata dose di farmaco, e si osserva inoltre diminuita la coagulabilità del sangue.

Questi fatti, come dissi in principio della mia tesi, stanno in rapporto con le ben note interferenze che sussistono fra ioni calcio e fosforo, studiate così profondamente da SABBATANI.

Con la dose minima letale lontana, come con quelle immediatamente superiori, ciò che domina il quadro clinico ed anatomo-patologico è il forte dimagramento generale e la consecutiva atrofia del fegato. Si deve ammettere perciò che il farmaco abbia agito profondamente sui centri del metabolismo organico degli animali che non sono più in grado di mantenere in pareggio il loro ricambio materiale, pur assumendo una quantità di cibo normale. La morte in questo caso è dovuta appunto alle alterazioni metaboliche ed alla conseguente cachessia.

Oltre a ciò bisogna tener presente le alterazioni a carico dei reni, consistenti in una nefrosi più o meno grave a seconda della dose del farmaco iniettato, lieve con le dosi piccole, molto intensa con le dosi alte. Se le lievi alterazioni renali degli esperimenti VII ed VIII non possono avere che importaza secondaria per la morte dell'animale, le alterazioni renali più gravi delle esperienze V e VI possono far pensare che la morte di questi due conigli sia dipendente anche dalle alterazioni nefrotiche e dalla conseguente intossicazione uremica dell'organismo.

CONCLUSIONI.

In conclusione dunque dalle mie ricerche risulta che:

I - La dose minima mortale lontana per via endovenosa nel coniglio è uguale a gr. 1,075 di fosfato bisodico per Kg. (gr. 0,093 di fosforo per Kg).

II - La quantità di fosfato bisodico che provoca i primi accessi tetano-simili nel coniglio (il « livello tetanico ») è uguale a 1,444 per Kg. (gr. 0,125 di fosforo per Kg.).

III - Con la dose minima letale e con quelle immediatamente superiori la morte interviene in seguito alla cachessia dovuta alle alterazioni profonde dei centri del metabolismo.

IV - Con dosi elevate la morte avviene per le alterazioni chimiche e chimico-fisiche del sangue e dei tessuti, specie dei centri nervosi (tetania).

V - Con le dosi intermedie fra le precedenti nel meccanismo della morte intervengono le alterazioni nefrotiche gravi dei reni.

VI - Con le dosi inferiori alla minima mortale lontana gli animali sopravvivono e reagiscono all'introduzione del farmaco soltanto con una transitoria diminuzione di peso, tanto più leggera quanto più piccola fu la dose del farmaco introdotta.

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RIASSUNTO

Il fosfato bisodico iniettato nelle vene dei conigli a dosi elevate uccide l'animale con delle contrazioni caratteristiche simili a quelle del tetano. A dosi minori uccide con una sindrome depressiva costante accompagnata da profondo dimagrimento se l'animale sopravvive qualche giorno. La dose minima letale lontana è di gr. 1.075 pro chilo.

RÉSUMÉ

Le Phosphate disodique injècté dans les veines des lapins à doses élevées tue animal avec de contractions caractéristiques ressemblantes aux tétany doses minuers tue avec une sindrome depressive constante accompagnée d'un profonde amaigrément, lorque l'animal survit pendant quelques jours. La dose minime letale a distance est dans le lapin de g. eq. 1,075 par Kgr.

ZUSAMMENFASSUNG

Wenn man saures Natriumphosphat in die Venen der Kaninchen zu hohen Dosen einspritzt, so tötet dieses das Tier unter charakteristischen Zusammenziehungen wie beim Starrkrampf. Zu kleineren Dosen tötet es durch konstante depressiven Erscheinungen die von ausserordentlicher Abmagerung begleitet sind, wenn das Tier einige Tage überlebt. Die minimale tödliche Dosis ist 1,075 pro chilo.

SUMMARY

The acid sodium phosfate when intravenously injected in high doses in the rabbits, causes the dead of the animal under characteristic Tetanus - like contractions. When smaller doses ace used the animal dies under a constant depressive syndrome. If the animal con be alive for some dags a veny deep leanness is observed.

The minimal lethal dose is eq. gr. 1,075 pro Kilogram.

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METABOLIC STUDIES IN PATIENTS WITH HYPERCALCIURIA

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THE association between a high excretion of calcium in the urine and the formation of calcium-containing renal stones is well known (Flocks, 1939), but careful studies have failed to establish a definite relationship between the two. One difficulty has been the lack of a clear cut separation of hypercalciuric from normocalciuric individuals. Hodgkinson and Pyrah (1958) have suggested a normal range for urine calcium excretion of 100 to 300 mg./24 hours for males and 100 to 250 mg./24 hours for females on a normal diet, and by this criterion about 8% of the general population have an abnormally high calcium excretion. Hypercalciuria is common in primary hyperparathyroidism, renal tubular acidosis, sarcoidosis, Cushing's syndrome, Paget's disease, Vitamin D intoxication, thyrotoxicosis, acromegaly, myelomatosis and Fanconi syndrome, but renal stone formation occurs with significant frequency in only the first four conditions. From patients presenting with primary calcium-containing renal stones Melick and Henneman (1958) found that only 32% had hypercalciuria whilst Hodgkinson, Purton and Pyrah (1961) found that, depending on the crystallographic appearance of the stone, between 15% and 53% were hypercalciuric. The majority of patients with renal stones and hypercalciuria have no detectable primary disease. A group of them studied by Albright, Henneman, Benedict and Forbes (1953) were found to be predominantly male, have a low blood phosphorus and have Staphylococcus albus infection in the urine. It was suggested that the condition might represent a clinical entity which they called "Idiopathic Hypercalciuria". However, a disorder defined principally by exclusion is unlikely to be homogeneous and since stone formers are predominantly male, and may show a low blood phosphorus whatever the level of calcium excretion (McGeown, 1957) and the high incidence of Staphylococcus albus infection has not been confirmed (Harrison, 1959) it is difficult to accept that the findings establish a clinical disease. It is, however, convenient to retain the term idiopathic hypercalciuria but without any nosological overtones.

This paper concerns the study of ten patients suffering from recurrent renal stone formation and hypercalciuria. It was the purpose of the investigation to establish, if possible, a known cause for the hypercalciuria in each case and to study means by which the renal calcium excretion might be effectively reduced.

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TABLE I

Summary of the salient investigations

Case	Sex	Diagnosis	Dietary calcium mg./day	X-ray renal tract	Stone analysis	Blood						Urine				
						Ca mg.%	P mg.%	Phos KA units	CO_2 mEq./l.	Urea mg.%	Plasma proteins g.%	Pus cells	Organisms	Urea clearance	Max. S.G.	Min. pH
1	M	Idiopathic hypercalciuria	1300	Normal	Ca HPO_4	9·8	3·8	9·1	29·3	34	6·3	Few	S. fæc. E. coli	—	1026	5·56
2	M	Idiopathic hypercalciuria	1500	Bilateral stones	—	9·3	3·5	8·6	28·4	21	6·7	None	None	98	1012	—
3	M	Idiopathic hypercalciuria	770	Bilateral stones Nephrocalcinosis	—	11·0	3·8	7·6	25·8	24	7·2	Few	S. fæc. E. coli	78	1012	5·9
4	M	Idiopathic hypercalciuria	820	Bilateral stones	Ca HPO_4	9·2	3·2	9·7	27·2	24	6·8	Few	S. albus	93	1012	4·9
5	M	Idiopathic hypercalciuria	860	Bilateral stones Right bifid pelvis	Ca HPO_4 Oxalate Mg NH_4	9·1	3·6	7·0	25·3	31	6·7	Few	E. coli	—	1016	4·9
6	F	Primary hyperparathyroidism	650	Left staghorn calculus. Right small stone	Ca HCO_3 Oxalate HPO_4	11·1	3·1	8·1	24·0	23	6·7	Many	None	71	1016	—
7	M	Primary hyperparathyroidism	—	Normal	—	10·8	3·6	5·2	31·3	27	8·0	None	None	—	1020	—
8	M	Primary hyperparathyroidism	840	Bilateral stones	Ca HPO_4	10·4	2·8	6·2	28·9	24	7·1	None	None	—	1014	5·3
9	M	Sarcoidosis	880	Left sided stones	Ca HPO_4	9·4	3·8	35·0	25·5	28	8·1	None	None	62	1016	5·9
10	M	Paget's disease	670	Normal	Ca HPO_4 Mg NH_4	9·6	3·1	23·7	25·3	38	5·9	None	None	—	1034	5·3

METHODS

All patients were admitted to the Metabolic Ward of the Royal National Orthopædic Hospital. None was confined to bed except for intercurrent illness. In addition to routine investigations (Table 1) the following tests were carried out. Water conservation was measured during fluid deprivation from 10.00 p.m. to midday the following day and a minimum specific gravity above 1·020 was considered normal. Urine acidification was investigated by a modification of the short test outlined by Wrong and Davies (1959). At 9.00 a.m., 5·0 g. ammonium chloride was given to the fasting subject, and urine pH and the rate of ammonia production were measured at hourly intervals on freshly passed specimens over the subsequent four to six hours. A pH of 5·3 or less was taken as a normal response. The rate of ammonia production was plotted on a logarithmic scale against urine pH (Wrong and Davies, 1959). In cases where the serum calcium level was doubtfully raised, plasma calcium fractionation (Rose, 1957) was performed at University College Hospital. Paper chromatography was used for the detection of urine amino acids (Smith, 1958).

Metabolic balances for calcium, phosphorus and nitrogen were carried out according to the principles of Reifenstein, Albright and Wells (1945). A detailed description of the procedure followed has been given in a previous paper from this Unit (Walker and Collins, 1963). Serum calcium was estimated by the method of Fales (1953); normal range 9·5–10·5 mg./100 ml.

When substances containing extra phosphorus were given, the amount was determined by separate analysis and added to the amount in the diet. Neutral sodium phosphate was given as a mixture of 3·64 parts Na_2HPO_4 to one part $NaH_2PO_4.2H_2O$ containing 21·4% phosphorus. The sodium phytate used in Studies 2, 3, 4 and 10 was obtained from Ciba Ltd., Horsham, Sussex, and contained 18·9% total phosphorus, 1·1% of which was inorganic phosphate, and that used in Studies 5, 8 and 9 from Brown and Polson Ltd., London, W.C.2, contained 18·2% total phosphorus, 2·8% of which was inorganic phosphate. Cellulose phosphate was obtained from Reeve, Angel and Co. Ltd., London, E.C.4. That used in Studies 5 and 9 was supplied in the ammonium form and converted to the sodium form by repeated washing with dilute HCl until the wash was ammonia free and then adding dilute NaOH to pH 7·0. After drying it contained 8·2% total phosphorus. That used in Study 8 was supplied by the manufacturers in the sodium form and contained 9·3% total phosphorus.

RESULTS

Of the ten patients studied in the investigation, three (cases 6, 7 and 8) were found to be suffering from primary hyperparathyroidism (proven at operation), one from sarcoidosis (Case 9) and one from Paget's disease (Case 10). In Case 10 it was not easy to decide to what extent Paget's disease alone was responsible for the hypercalciuria because the disease was localised to only two vertebral bodies and the plasma alkaline phosphatase was normal. In the remaining five cases (Cases 1, 2, 3, 4 and 5) no cause for the hypercalciuria could be found and these have been labelled as idiopathic hypercalciuria. The case histories are given in an appendix and salient points from the preliminary investigations are given in Table 1. All the patients presented in a similar manner, that is with renal stones, and clinically there was no obvious difference between them. Normal serum calcium values were initially found in two of the patients with parathyroid adenoma (Cases 7 and 8) and only subsequently were increased values found. Serum phosphorus levels were of no help in diagnosis, being initially low in only one patient with primary hyperparathyroidism and varying considerably between normal and low on repeated examinations. No significant proteinuria was found. Positive cultures were found at some time in four patients with idiopathic hypercalciuria, but no organism predominated and Staphylococcus albus was found only once. Impaired water conservation was found in four patients with idiopathic hypercalciuria and in three others. The plasma CO_2 combining power and blood urea were normal in all cases, but urea clearance was slightly impaired in Case 9. In the seven cases in which urine acidification was studied three subjects failed to acidify to a pH below 5·3 (Cases 1, 3 and 9), but the rate of ammonia production was normal in all cases. Examination of the urine for abnormal amino acid content was negative in every case.

The metabolic data is summarised in Table 2. Figs. 1–10 are metabolic charts for calcium, phosphorus and nitrogen constructed according to the method of Albright and Reifenstein (1948) (see legend to Fig. 1). In interpreting the results

TABLE 2A

Summary of balance data

Patient	Regime	Days	Calcium mg./day				Phosphorus mg./day				Nitrogen g./day			
			Intake	Urine	Fæces	Bal.	Intake	Urine	Fæces	Bal.	Intake	Urine	Fæces	Bal.
Case 1 R.P.	Control	6	786	259	564	−37	1131	975	369	−204	10·95	10·60	2·17	−1·82
	Control	18	,,	318	518	−50	,,	980	339	−188	,,	10·46	2·02	−1·53
	Cortisone 200 mg./day	12	,,	416	458	−88	,,	924	377	−170	,,	11·47	1·79	−2·31
	Control	6	,,	435	553	−182	,,	1120	371	−368	,,	11·10	1·83	−1·98
Case 2 R.D.	Control	12	945	461	626	−141	1390	1081	451	−142	15·64	14·15	1·90	−0·41
	Ammonium chloride 8 g./day—3 days	6	,,	542	680	−277	,,	985	606	−201	,,	—	2·11	—
	Cortisone 150 mg./day	12	,,	452	558	−65	,,	1040	430	−80	,,	15·20	1·39	−0·95
	Sodium phytate 8 g./day	24	,,	277	878	−210	2902	*1654	*1407	−159	,,	14·27	2·16	−0·79
	Low calcium intake	18	384	359	264	−239	954	898	304	−248	13·20	13·41	1·64	−1·85
Case 3 T.G.	Control	18	870	525	491	−146	1215	1082	233	−100	12·25	10·88	1·65	−0·28
	Cortisone 150 mg./day	12	,,	584	415	−129	,,	981	225	+9	,,	11·84	1·62	−1·21
	Control	6	,,	451	482	−63	,,	1180	258	−203	,,	12·54	2·15	−2·44
	Sodium bicarbonate 10 g./day	18	,,	420	485	−35	,,	948	287	−20	,,	9·79	2·21	+0·25
	Sodium phytate 6 g./day	6	,,	288	569	+13	2349	1288	959	+102	,,	10·15	1·93	+0·17
	Sodium phytate 8 g./day	12	,,	262	513	+95	2727	1615	1049	+63	,,	10·37	1·90	−0·02
	Control	6	,,	484	445	−59	1215	1105	301	−191	,,	12·35	1·72	−1·82
	Sodium phosphate 6·26 g./day	18	,,	376	444	+50	2555	1869	489	+197	,,	11·07	1·83	−0·65
Case 4 W.M.	Ammonium chloride 8 g./day—3 days	6	840	640	727	−527	1360	1233	428	−301	14·15	16·50	2·42	−4·77
	Control	12	,,	517	453	−130	,,	1014	328	+18	,,	12·66	2·65	−1·21
	Sodium bicarbonate 10 g./day	18	,,	372	496	−28	,,	986	303	+71	,,	12·01	2·25	−0·12
	Control	6	,,	446	396	−2	,,	1105	242	+13	,,	12·87	1·87	−0·59
	Cortisone 150 mg./day	18	,,	577	395	−132	,,	1048	330	−18	,,	13·48	2·12	−1·45
	Control	12	,,	475	464	−104	,,	1080	327	−47	,,	12·56	2·55	−0·96
	Sodium phytate 6 g./day	6	,,	301	555	−16	2494	1207	1110	+177	,,	13·38	2·20	−1·43
	Sodium phytate 8 g./day	12	,,	288	538	+14	2872	1512	1305	+55	,,	11·19	2·43	+0·53
	Control	12	,,	496	469	−125	1360	1036	409	−85	,,	13·27	2·28	−0·40
	Sodium phosphate 9·39 g./day	6	,,	325	380	+135	3370	2320	576	+474	,,	11·80	1·92	+0·43
	Sodium phosphate 6·26 g./day	12	,,	355	452	+32	2700	1965	462	+273	,,	12·49	2·24	−0·58
Case 5 A.L.	Control	18	871	369	598	−96	1165	888	375	−98	12·70	11·39	1·97	−0·66
	Sodium bicarbonate 10 g./day	6	,,	320	502	+49	,,	878	344	−57	,,	10·82	1·86	+0·02
	Cortisone 150 mg./day	12	,,	478	516	−123	,,	845	358	−38	,,	12·04	1·56	−0·90
	Control	6	,,	410	674	−213	,,	1015	523	−373	,,	11·40	1·92	−0·62
	Sodium phytate 6 g./day	6	,,	295	460	+116	†2294	1338	802	+154	13·43	12·40	1·87	−0·84
	Sodium phytate 8 g./day	12	,,	226	671	−26	2658	1507	1076	+76	,,	11·52	2·43	−0·51
	Sodium phosphate 6·26 g./day	6	,,	228	746	−103	2542	1624	686	+232	,,	11·48	2·28	−0·33
	Cellulose phosphate 15 g./day	12	,,	247	683	−59	2432	985	1417	+30	,,	12·30	2·21	−1·08

* Six days only.
† Dietary phosphorus increased by 37 mg.

TABLE 2B

	g./day													
	Cortisone 150 mg./day	12	"	478	516	−123	"	845	358	−38	"	12·04	1·56	−0·90
	Control	6	"	410	674		"	1015	523	−373	"	11·40	1·92	−0·62
	Sodium phytate 6 g./day	6	"	295	460		†2294	1318	802	+154	13·43	12·40	1·87	−0·84
	Sodium phytate 8 g./day	12	"	226	671		2658	1507	1076	+76	"	11·52	2·43	−0·51
	Sodium phosphate 6·26 g./day	6	"	228	746		2542	1624	686	+232	"	11·48	2·28	−0·33
	Cellulose phosphate 15 g./day	12	"	247	683		2432	985	1417	+30	"	12·30	2·21	−1·08

* Six days only.
† Dietary phosphorus increased by 37 mg.

TABLE 2B

Summary of balance data

Patient	Regime	Days	Calcium mg./day				Phosphorus mg./day				Nitrogen g./day			
			Intake	Urine	Fæces	Bal.	Intake	Urine	Fæces	Bal.	Intake	Urine	Fæces	Bal.
Case 6 E.L.	Control	12	697	383	512	−198	1033	670	346	+17	11·80	10·09	1·46	+0·25
	Cortisone 150 mg./day	12	"	379	581	−213	"	729	362	−58	"	9·57	1·53	+0·70
	Control	6	"	386	434	−123	"	970	292	−229	"	12·03	1·21	−1·45
Case 7 T.R.	Control	6	420	211	383	−174	957	625	262	+60	10·80	8·44	1·14	+1·22
	Control	12	"	198	308	−86	"	719	234	+5	"	9·63	1·14	+0·03
	Cortisone 150 mg./day	12	"	249	246	−75	"	764	218	−25	"	11·11	1·15	−1·46
	Control	6	"	194	243	−17	"	920	239	−202	"	11·50	1·22	−1·92
Case 8 K.T.	Control	6	864	396	436	+32	1436	967	520	−51	13·50	11·10	2·72	−0·32
	Control	6	850	388	524	−62	1311	955	442	−86	13·45	11·20	2·21	+0·04
	Sodium bicarbonate 12 g./day	6		310	579	−39	"	900	542	−131	"	11·35	2·23	−0·13
	Sodium phytate 6 g./day	6	"	244	568	+38	2403	1450	787	+166	"	10·83	1·88	+0·74
	Sodium phytate 8 g./day	6	"	264	755	−169	2767	1785	1204	−222	"	10·35	2·86	+0·24
	Sodium phosphate 6·26 g./day	6	"	288	498	+64	2651	1865	773	+13	"	10·66	2·28	+0·51
	Cellulose phosphate 15 g./day	12	"	281	580	−11	2698	1157	1353	+188	"	11·08	2·25	+0·12
	Cortisone 150 mg./day	6	"	435	454	−49	1311	1033	410	−131	"	12·26	1·83	−0·61
Case 9 M.H.	Control	18	805	356	531	−82	1220	895	364	−39	13·35	11·68	1·76	−0·09
	Sodium bicarbonate 15 g./day	6	"	240	632	−67	"	742	600	−122	"	11·43	1·93	−0·01
	Cellulose phosphate 15 g./day	6	"	199	672	−66	2450	1024	1206	+220	"	11·30	2·20	−0·15
	Control	6	"	305	558	−58	1220	937	352	−69	"	12·63	1·90	−1·18
	Sodium phytate 7 g./day	6	"	193	654	−42	2495	1325	960	+210	"	11·10	1·64	+0·61
	Cortisone 150 mg./day	12	"	264	790	−249	1220	840	667	−257	"	13·29	1·78	−1·72
	Cellulose phosphate 15 g./day	12	"	237	702	−134	2450	986	1497	−33	"	12·26	2·08	−0·99
	Cellulose phosphate and sodium bicarbonate 15 g./day	6	"	178	689	−60	"	806	1813	+131	"	10·22	2·04	+1·09
	Calciferol 0·5 mg./day	6	"	319	539	−53	1220	797	368	+55	"	10·70	1·65	+1·00
Case 10 F.B.	Ammonium chloride 8 g./day – 4 days	6	702	694	517	−509	1220	1120	259	−159	13·80	13·95	1·33	−1·45
	Recovery	6	"	520	404	−222	"	915	297	+8	"	12·76	1·38	−0·34
	Control	18	"	370	453	−121	"	871	341	+8	"	11·74	1·70	+0·36
	Cortisone 150 mg./day	12	"	400	299	+3	"	905	285	+30	"	12·97	1·76	−0·93
	Control	6	"	424	283	−5	"	881	248	+91	"	10·86	2·48	+0·76
	Sodium bicarbonate 10 g./day	12	"	360	357	−15	"	805	287	+128	"	9·45	1·92	+2·43
	Sodium phytate 6 g./day	6	"	177	357	+168	2345	1035	976	+343	"	9·71	1·90	+2·39
	Sodium phytate 8 g./day	18	"	176	524	+2	2732	1265	1182	+283	"	10·64	1·97	+1·19
	Control	6	"	376	462	−136	1220	916	511	−207	"	11·43	2·14	+0·23
	Sodium phosphate 6·26 g./day	18	"	309	440	−47	2560	1567	665	+328	"	10·54	2·18	+1·08

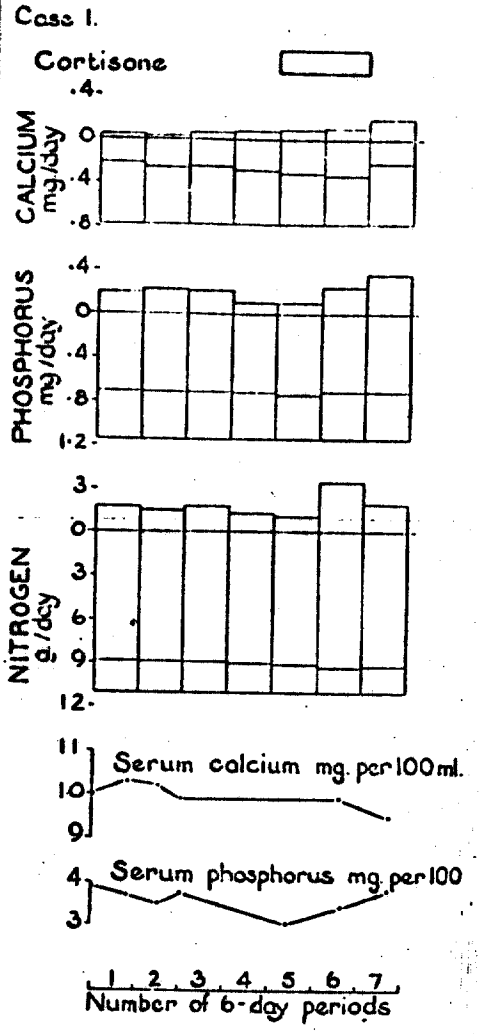

Fig. 1.

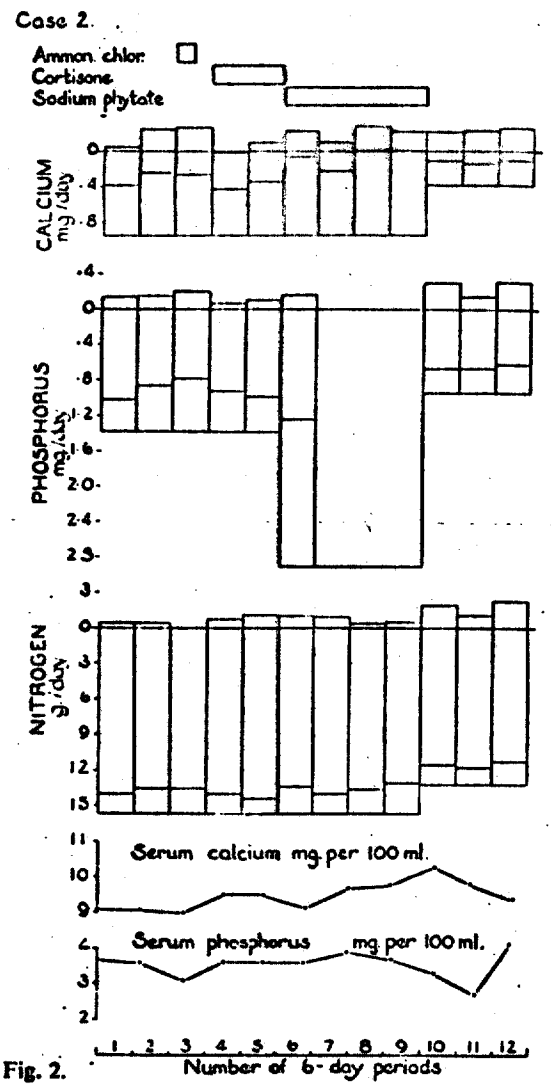

Fig. 2.

Fig. 1. *Case 1—Idiopathic hypercalciuria.*

The calcium intake of 786 mg./day was substantially lower than the previous intake of 1,300 mg./day. This may explain the progressive fall in faecal calcium in control periods 1–4. The urine calcium in Period 1 may have been lowered by an attack of renal colic from which the patient was just recovering. For these reasons Period 1 has been disregarded in calculating the response to cortisone. There was no fall in urine calcium in the post-cortisone period.

The following method is used for the charting of calcium, phosphorus and nitrogen balances in Figs. 1–10. Each block represents a six-day collection of urine and fæces and a gap indicates that periods were not consecutive. The average daily intake is plotted down from the zero line and from this baseline the faecal calcium (lower block) then the urine calcium (upper block) are plotted back up towards the zero line. An area appearing above the zero line represents an excess of output over intake (negative balance) and a gap below the zero line represents an excess of intake over output (positive balance).

Fig. 2. *Case 2—Idiopathic hypercalciuria.*

The calcium intake of 945 mg./day was substantially lower than the intake over the years of 1,500 mg./day but approximated that taken recently on medical advice. The study was marred by irregular faecal demarcation, indicated by the fluctuating faecal nitrogen excretion. Ammonium chloride was given for three days only and the increase in nitrogen of 1·04 g./day is not shown. Unfortunately no control period was interpolated between this period and the next on cortisone.

of the metabolic studies, changes occurring in the experimental periods have been evaluated by comparison with findings in the initial control periods. Since many of the studies extended for several months, this is not entirely satisfactory but when control periods were interpolated between experimental periods, the effects of the previous regime were usually still apparent. Ideally, sufficient time should elapse between each regime to observe a return to the baseline conditions, but this would have prolonged the studies beyond the endurance of the patients.

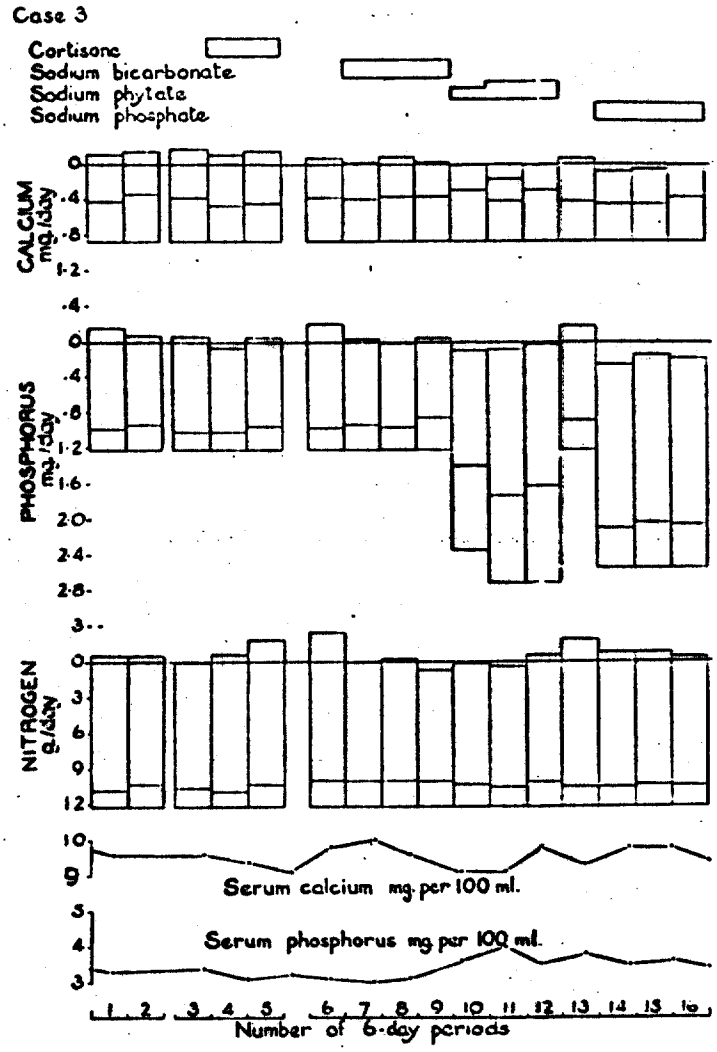

Fig. 3. *Case 3—Idiopathic hypercalciuria.*
Periods 2 and 3 and Periods 5 and 6 were separated by gaps of two days during which special tests were carried out. Not explored because all subsequent calcium estimations were normal.

All the patients were in a slightly negative calcium balance of from 50 to 198 mg./day. According to the criteria of Hodgkinson and Pyrah (1958) all had hypercalciuria except Case 7. In Case 7 two twenty-four hour urine calcium

estimations done soon after admission were 306 and 308 mg./day and the lower urine calcium found during balance was probably due to a marked reduction in the calcium content of the diet selected. Normal standards for faecal calcium excretion are difficult to define precisely. In a normal adult who is in zero balance for calcium, changes in urine and faecal calcium vary inversely. By substracting the faecal calcium from the intake a theoretical urine calcium necessary to give a zero balance can be obtained. If this figure is high the observed faecal calcium must be low or vice versa. In patients 2, 3 and 4 this theoretical urine calcium according to our previous standards of normality (below 300 mg./day for males and 250 mg./day for females) was undoubtedly raised and in the other two (1 and 5) it was near the upper limit of normal. Except for Case 7, the remainder of the patients with hypercalciuria due to known causes also had values above or near the upper limit of normal. By these criteria low faecal calcium was therefore present in nearly every case.

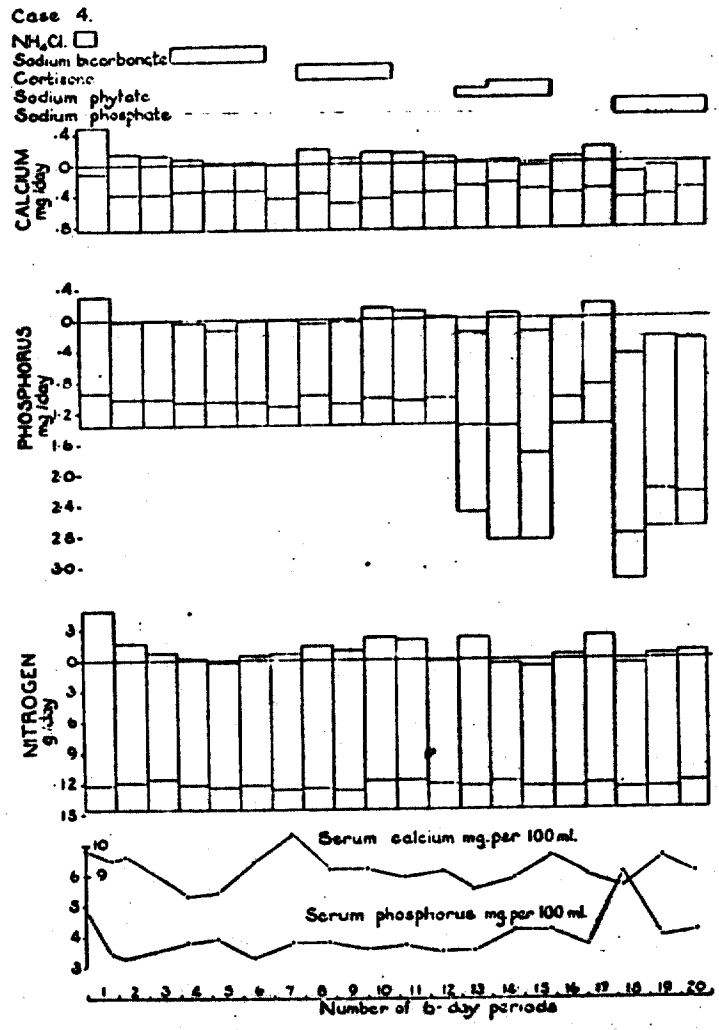

Fig. 4. *Case 4—Idiopathic hypercalciuria.*
Increase in nitrogen intake of 1·04 g./day due to ammonium chloride in Period 1 is not shown. An excessive dose of neutral sodium phosphate (9·39 g./day) was given by mistake in Period 18.

The effects of changes in acid/base balance on the calcium and phosphorus balances are summarised in Table 3. Ammonium chloride (8 g. daily for three days of a six day period) produced an increase of urine calcium in all three cases tested (Figs. 2, 4 and 10), a significant increase in faecal calcium in one case (4) and a negative balance in all. There was an increase in urine phosphorus and nitrogen

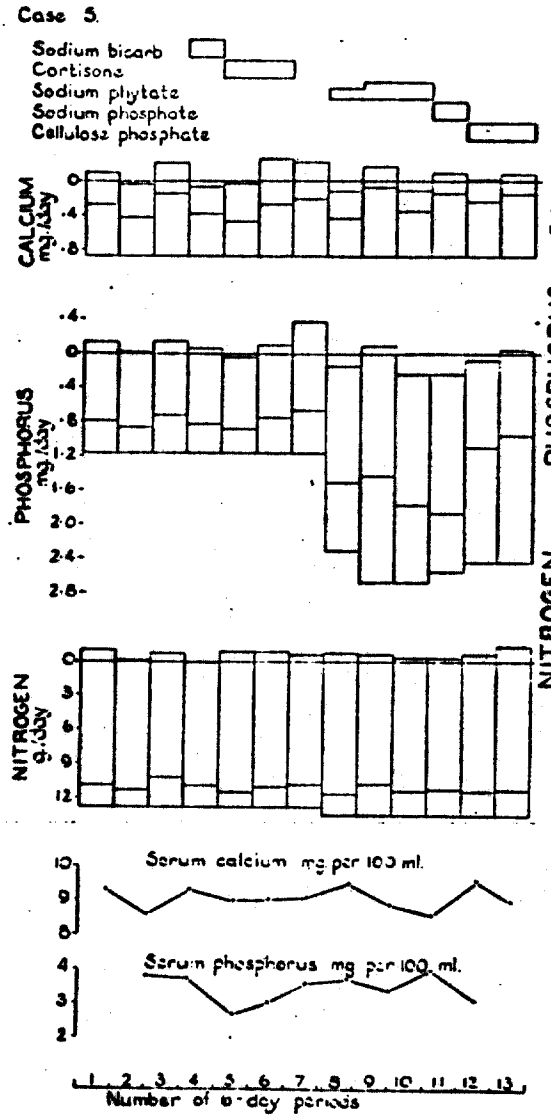

Fig. 5.

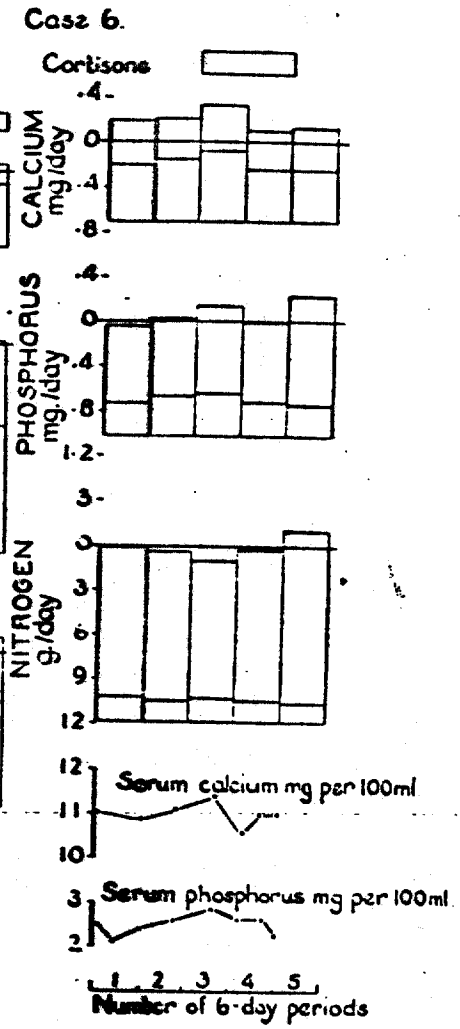

Fig. 6.

Fig. 5. *Case 5—Idiopathic hypercalciuria.*
Irregular faecal demarcation, indicated by fluctuation in faecal nitrogen, accounts for some but not all of the variations in faecal calcium. The patient had serious social problems, was a pethidine addict and was frequently unco-operative. The changes in urine calcium agree with those of the previous studies.

Fig. 6. *Case 6—Primary hyperparathyroidism.*
Serum calcium raised on several occasions.

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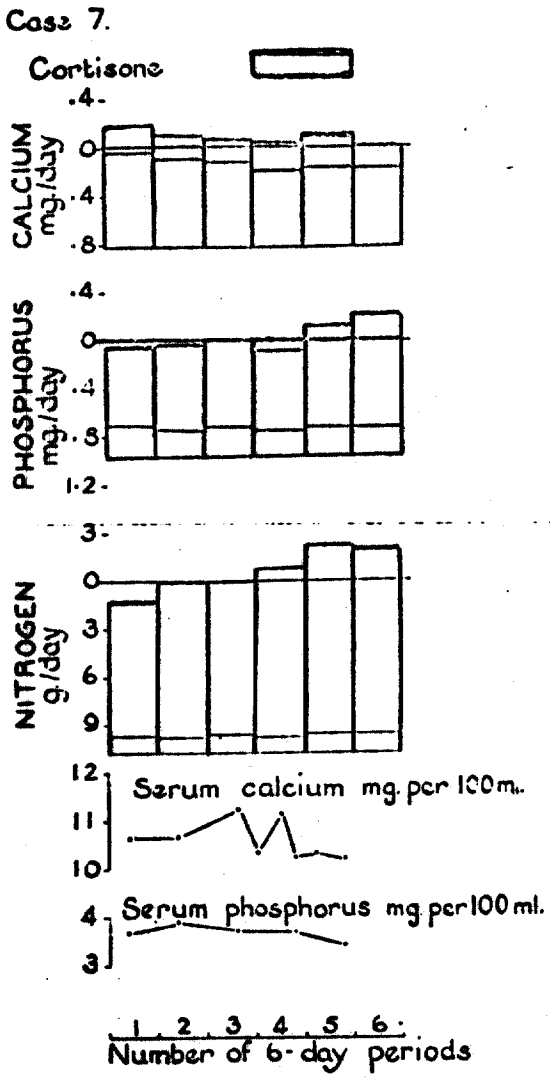

Fig. 7.

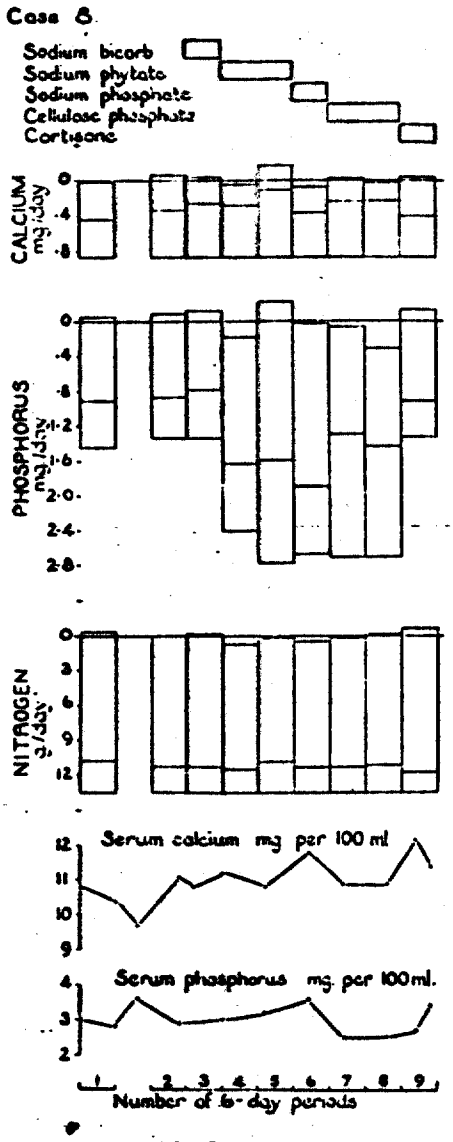

Fig. 8.

Fig. 7. *Case 7—Primary hyperparathyroidism.*

The changes in fæcal calcium in Periods 1–3 suggest that the actual recent intake was greater than prescribed and that the period was adapting to a lower intake. For this reason Period 1 has been disregarded from the calculations.

Fig. 8. *Case 8—Primary hyperparathyroidism.*

The patient had to leave hospital for personal reasons after Period 1. He was later re-admitted and re-equilibrated on a slightly different diet. Period 2 has been used as the control. The rise in urine phosphorus in Period 7 was probably due to excretion of phosphorus retained in Period 6. The fæcal phosphorus in Period 8 on cellulose phosphate was unexpectedly low, with an impossible degree of phosphorus retention. It is likely that the phosphorus intake was smaller than was expected. This study suffers from insufficient control periods, both initially and between the different regimes.

Fig. 9. *Case*

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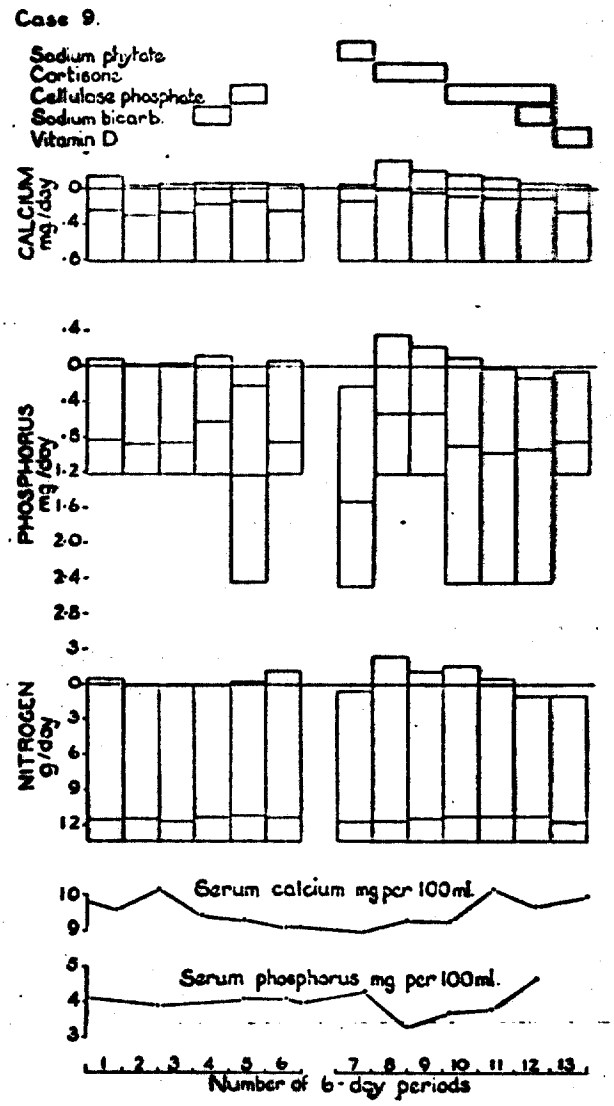

Fig. 9. *Case 9—Sarcoidosis.*

Period 5 was the first occasion in which cellulose phosphate was used in man. In this period there was an impossible degree of phosphorus retention with no change in the calcium or nitrogen balance. It is likely that, as in Case 8, that phosphorus intake was smaller than expected.

with a more negative balance in two cases (4 and 10). Sodium bicarbonate (10 g. daily) caused a fall in urine calcium of 49 to 145 mg./day in five cases (Figs. 3, 4, 5, 8 and 9). In Case 10 (Fig. 10) the fall was 64 mg./day if period 8 is taken as the control. There was also a slight fall in urine phosphorus and in urine nitrogen, except in Case 8. No significant change in fæcal calcium, phosphorus or nitrogen occurred. Both the patients with idiopathic hypercalciuria and with hypercalciuria due to known causes responded in a similar manner.

TABLE 3

The effect of changes in acid/base balance on calcium and phosphorus balance. The mean changes in urine (U), fæces (F) and overall balance (B) in the different groups of cases are derived from data in Table 2

Treatment	Diagnosis	No. of cases	Calcium mg./day			Phosphorus mg./day		
			U	F	B	U	F	B
Ammonium chloride 8 g./day	Idiopathic hypercalciuria	2	+102	+164	−258	+123	+128	−209
Ammonium chloride 8 g./day	Paget's disease	1	+324	+64	−388	+249	−82	−167
	Overall mean		+176	+131	−301	+124	+58	−195
Sodium bicarbonate 10 g./day	Idiopathic hypercalciuria	3	−100	−20	+119	−57	0	+58
Sodium bicarbonate 10 g./day	Primary hyperparathyroidism	2	−97	+78	+19	−104	+168	−64
Sodium bicarbonate 10 g./day	Paget's disease	1	−10	−96	+106	−66	−54	+120
	Overall mean		−84	0	+84	−74	+47	+28

The effects of cortisone 150 mg./day are summarised in Table 4. In Case 9, the patient with sarcoidosis, cortisone produced a well marked fall in urine calcium and a greater rise in fæcal calcium to produce a more negative calcium balance. A similar change occurred in the phosphorus balance (Fig. 9). In all the other patients there was a small increase in urine calcium of about 50 mg./day, except in Cases 2 and 6, where no change occurred, and a slight fall in fæcal calcium without alteration in the overall balance. The apparent lack of response in Case 2 may have been due to the interposition of the period on ammonium chloride between the control periods and the periods on cortisone (Fig. 2). In the post-cortisone control period in Cases 3, 4 and 5 the urine calcium fell by 133, 102 and 68 mg./day. The changes in phosphorus balance were variable. A more negative nitrogen balance, due to an increase in urine nitrogen occurred in all patients, except Case 6.

Table 5 summarises the effects of sodium phosphate, sodium phytate and cellulose phosphate on the calcium and phosphorus balances. Neutral sodium phosphate was given in a dosage of 6·26 g./day. This dose contains approximately

TABLE 4

The effect of cortisone on calcium, phosphorus and nitrogen balance The mean changes in urine (U), fæces (F) and overall balance (B) are derived from the data in Table 2

Diagnosis	No. of cases	Calcium mg./day			Phosphorus mg./day			Nitrogen g./day		
		U	F	B	U	F	B	U	F	B
Idiopathic hypercalciuria	5	+63	−69	+5	−41	−1	+23	+0·70	−0·37	−0·55
Primary hyperparathyroidism	3	+31	−38	+3	+61	−11	−50	+0·67	−0·07	−0·56
Paget's disease	1	+30	−154	+124	+34	−56	+22	+1·23	+0·06	−1·29
Sarcoidosis	1	−92	+259	−168	−55	+303	−218	+1·61	+0·02	−1·63

the same amount of phosphorus as 7 g. of sodium phytate. In Case 4 a larger dose of 9·39 g./day was given by mistake. A consistent fall in urine calcium (average 126 mg./day) occurred in every case. Excluding Case 5 (see legend Fig. 5) there was no change in the fæcal calcium and a positive calcium balance occurred (best shown in Figs. 3, 4 and 8). Approximately 81% (75–89%) of the extra phosphorus was absorbed. From this about 24% was retained in the body (295 mg./day or 16% of total extra intake) and the remainder excreted in the urine.

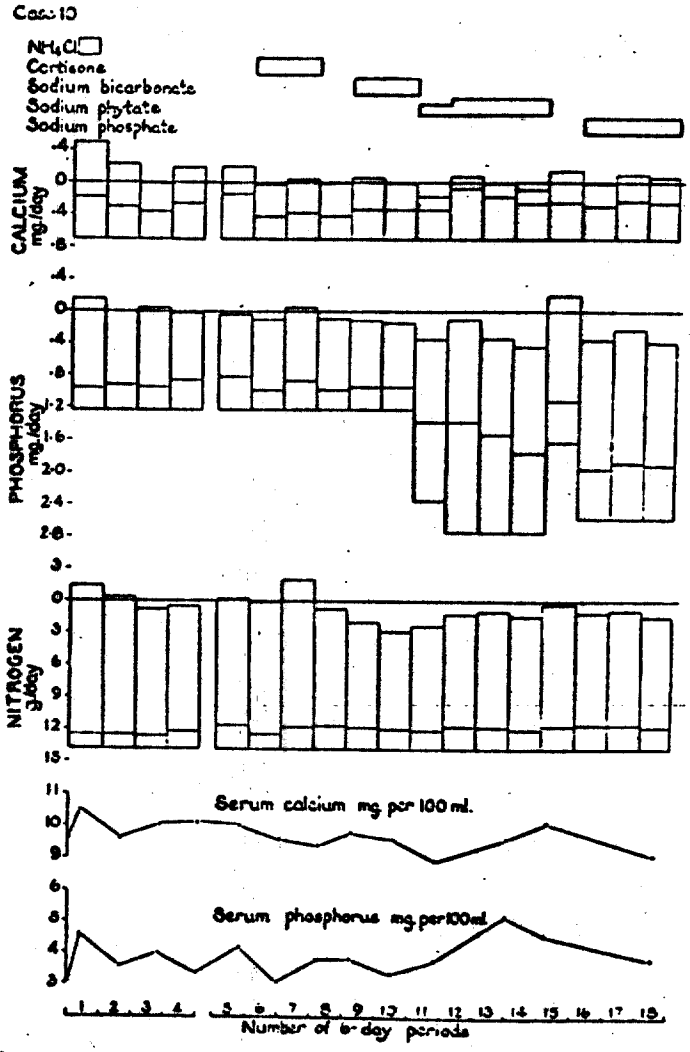

Fig. 10. *Case 10—Paget's disease and hypercalciuria.*
Ammonium chloride 8 g. daily was given on the first four days of Period 1. Period 2 is disregarded in calculating the averages because of the persistence of the effects of ammonium chloride.

Sodium phytate (6–8 g. daily) was given to seven patients. Mild diarrhœa occurred initially in each patient and this was not prevented by beginning with a smaller dose. A substantial fall in urine calcium (120–254 mg./day) occurred in all cases. The fall was greater on the larger dosage, except in Case 5. A clear cut

TABLE 5

The fate of the extra phosphorus given in the three different forms and the correlation with changes in the calcium balance. The mean change in mg./day in urine (U), fæces (F) and balance (B), phosphorus intake (I) and the percentage of extra phosphorus absorbed $\left(\frac{U + B}{I} \times 100\right)$ *(%A) are shown*

Case No.	Periods	Calcium mg./day			Phosphorus mg./day				
		U	F	B	I	U	F	B	%A
Sodium phosphate									
3	3	−149	−47	+196	1340	+787	+256	+297	81
4	3	−172	−25	+197	1563	+1069	+172	+322	89
5	1	−141	+148	−7	1340	+736	+311	+330	80
8	1	−100	−26	+126	1340	+910	+331	+99	75
10	3	−61	−13	+74	1340	+696	+324	+320	75
	Mean	−126	−12	+139	1401	+846	+263	+295	81
Sodium phytate									
2	1	−184	+252	−69	1512	+573	+956	−17	36
3	3	−254	+41	+213	1386	+424	+786	+176	43
4	3	−225	+91	+134	1386	+396	+912	+78	34
5	3	−120	+3	+117	1335	+563	+610	+199	51
8	2	−134	+138	−4	1274	+663	+554	+58	57
9	1	−163	+123	+39	1275	+430	+596	+249	53
10	4	−194	+29	+165	1415	+337	+790	+288	44
	Mean	−188	+69	+118	1372	+460	+750	+168	46
Cellulose phosphate									
5	2	−122	+85	+37	1230	+97	+1042	+128	18
8	2	−107	+56	+51	1387	+202	+911	+274	34
9	2	−119	+171	−53	1230	+91	+1133	+6	8
	Mean	−116	+104	+12	1282	+130	+1029	+136	21

rise in fæcal calcium occurred in five cases (2, 3, 4, 8 and 9). In four patients the fall in urine calcium clearly exceeded the rise in fæcal calcium and resulted in a more positive balance (Figs. 3, 4, 5 and 10). In two patients (Figs. 8 and 9) there was no significant change in the overall balance and in one case (Fig. 2) the reverse occurred with a more negative balance. The phosphorus balance showed that 34% to 57% of the extra phosphorus intake was absorbed. This phosphorus was handled in a similar manner to that described for sodium phosphate. Approximately 26% was retained and the rest excreted in the urine.

Cellulose phosphate was given to three patients. A fall in urine calcium of about 115 mg./day and a rise in fæcal calcium of similar magnitude without changes in the overall balance occurred (Figs. 5, 8 and 9). Discounting Case 8, where the phosphorus balance was spoilt by the close apposition of the period on sodium phosphate, only 11% of the extra phosphorus was absorbed and about 60% of this appeared in the urine.

Discussion

All the patients in the series were suffering from hypercalciuria and renal stone formation. In five of them a known cause of their condition was demonstrated but in the remainder no cause could be found. Analysis of the metabolic data shows no essential differences in the control periods between the patients in the known and unknown group and the ultimate diagnosis depended upon the results of other investigations.

The effects of alteration of acid/base balance on calcium metabolism in normal people has been studied by Farquharson, Salter, Tibbetts and Aub (1931), who found that ammonium chloride or an acid ash diet produced a rise in urine calcium

with a negative calcium balance, but that large amounts of sodium bicarbonate or sodium citrate produced no change. A similar lack of response to alkali therapy has been reported by Henneman, Benedict, Forbes and Dudley (1958) in one case of idiopathic hypercalciuria and by Howard, Parson and Bigham (1945) in cases of immobilisation hypercalciuria. Metabolic acidosis in our patients produced the expected response (Farquharson *et al.*, 1931) but sodium bicarbonate caused a fall in urine calcium similar to that described by Albright, Burnett, Parson, Reifenstein and Roos (1946) for cases of renal tubular acidosis. However, renal tubular acidosis, defined by these workers as a systemic metabolic acidosis and an inability to acidify the urine below a pH of 6, was not found in our patients. The incomplete form of renal tubular acidosis, described by Wrong and Davies (1959) was excluded by demonstration of a normal rate of ammonia production. However, these workers did describe two patients with hypercalciuria and nephrocalcinosis who resembled our cases (1, 3 and 9) in having some impaired power of acidification without systemic acidosis and normal ammonia production. The fall in urine calcium was not confined to the cases of idiopathic hypercalciuria and was of the same order in two patients who showed slightly abnormal ammonium chloride tests (Cases 3 and 9) as in the others. The findings could be explained by a renal tubular lesion caused by hypercalciuria itself which escapes detection because it functionally complies with the accepted standards of normality. Impairment of water conservation, another tubular function, was found in all except two of our patients and by Gill and Bartter (1961) in patients with hypercalciuria.

The response to cortisone clearly separated the patient with sarcoidosis (Case 9) from the other patients. Whereas in this patient there was a rise in fæcal calcium and fall in urine calcium, in the others a rise in urine calcium with a slight fall in fæcal calcium occurred. The response in sarcoidosis has also been reported by Anderson, Harper, Dent and Philpot (1954); Henneman, Dempsey, Carrol and Albright (1956). The findings indicate that our cases of idiopathic hypercalciuria are unlikely to be ætiologically related to sarcoidosis or hypersensitivity to Vitamin D. A small rise in urine calcium has also been reported by Pechet, Bowers and Bartter (1959) and Henneman *et al.* (1958) in normal people, and by Harrison (1959) and Henneman *et al.* (1958) in patients with idiopathic hypercalciuria. Although the changes in fæcal calcium were small in the individual cases (except Case 10) all showed a small decrease. The negative nitrogen balance found in all cases reflects the antianabolic effect of the corticosteroid drugs.

In the absence of bone disease the cause of the high urine calcium in cases of idiopathic hypercalciuria may be primarily renal or intestinal. Renal hypercalciuria could result from damage to the renal tubular re-absorptive mechanism for calcium, possibly following infection as suggested by Henneman *et al.* (1958). In these circumstances patients would be expected to be in a continuous negative calcium balance; the serum calcium would tend to fall, thus stimulating the parathyroid glands and giving a low serum phosphorus (Albright and Reifenstein, 1948). On the other hand, hypercalciuria might arise from intestinal over-absorption. Excessive quantities of calcium absorbed from the diet have to be excreted if the patient is to remain in zero balance. The consequent handling of large amounts of calcium by the kidney may lead to stone formation and possibly nephrocalcinosis. Accordingly the serum calcium would tend to be higher than average normal and the calcium balance zero. A slightly raised serum calcium has, in fact, been found by two groups of workers (Henneman *et al.*, 1958; Boyce and Bradshaw, 1961) but this is difficult to reconcile with the low serum phosphorus.

The low fæcal calcium often found in idiopathic hypercalciuria suggests over-absorption. Henneman *et al.* (1958) first drew attention to this point but all their patients were studied on a low calcium intake. Harrison (1959) reported two cases

as having normal fæcal calcium and negative calcium balance but a third case had a low fæcal calcium and a zero balance, as did a single case reported by Pyrah (1958). In our small series of cases some degree of increased calcium absorption was present in almost every case. However, Malm (1958) has shown that normal individuals after a moderate reduction of calcium intake are capable of considerably increasing their intestinal absorption of calcium. The low fæcal calcium of idiopathic hypercalciuria might then represent a secondary increase in calcium absorption following calcium loss via the kidney, as was first suggested by Jackson and Dancaster (1959). The state of the calcium balance should help to clarify this point. All the patients with idiopathic hypercalciuria were in a slight negative balance which favours a primary renal loss of calcium. However, the patient with sarcoidosis, where increased intestinal absorption is well known to occur was also in negative balance. Albright and Reifenstein (1948) suggested that in idiopathic hypercalciuries a negative calcium balance might lead to osteomalacia, but it is more likely that if bone disease developed it would take the form of osteoporosis (Nordin, 1961). None of our patients showed any evidence of bone disease, which is a point against any sustained negative calcium balance.

A sudden reduction in calcium intake should cause no change in a renal hypercalciuria, but a sharp reduction in hyperabsorption hypercalciuria. The response has been studied by several workers and variable results obtained. In some patients a good reduction in urine calcium occurs and in others very little change (Pyrah, 1958; Harrison, 1959; Boyce and Bradshaw, 1961; Litin, Diessner and Keating, 1961). The reason for this may be that idiopathic hypercalciuria comprises a heterogenous group of disorders of differing unknown ætiology, some of which are due to primarily renal causes and some to intestinal causes. Alternatively, a poor response to calcium deprivation does not necessarily exclude hyperabsorption, for as Malm (1958) has shown, many normal people can increase their absorptive capacity without necessarily altering their urine calcium. Such good adapters may, in fact, be hyperabsorbers but much further study on calcium balances in normal subjects and otherwise normal hypercalciuric subjects is required to decide this point. A further possibility may be that continued hypercalciuria may damage the renal tubular absorptive capacity for calcium, providing in many cases a combination of hyperabsorptive and renal hypercalciuria (Dent, personal communication). Other mild non-specific interference with tubular functions found in this series and the observations of Gill and Bartter (1961) would support this hypothesis.

The fall in urine calcium following sodium phosphate has been observed by Farquharson, Salter and Aub (1931) and Malm (1953) in normals; by Albright, Bauer, Claflin and Cockrill (1932) in hyperparathyroidism and by Saville, Nassim, Stevenson, Mulligan and Cary (1955) in resistant rickets That the fall is not due to an increased excretion of phosphorus by the kidney is shown by data obtained from phosphate infusions. Here a large acute increase in urine phosphorus produced by intravenous sodium phosphate leads to an increase in urine calcium excretion (Jahan and Pitts, 1948; Chen and Neuman, 1955). There was no change in fæcal calcium and a positive calcium and phosphorus balance occurred in every case. Phosphorus was probably retained partly in extra-cellular fluid, indicated by a rise in serum phosphorus (Figs. 4, 5 and 8); partly in bone, indicated by increased calcium retention (Figs. 3, 4, 8 and 10) and partly in protoplasm, indicated by increased nitrogen retention (Figs. 4, 5, 8 and 10), although certain discrepancies remain due to the limited accuracy of balance experiments. The fall in urine calcium may be due to a temporary increased deposition of bone salt, perhaps stimulated by a rise in blood phosphorus.

Phytic acid reduces the absorption of calcium from the intestine in experimental animals (Mellanby, 1950) and in humans (McCance and Widdowson, 1942), and

for this reason alone would be expected to lower urine calcium. However, some 45% of the extra phosphorus intake was absorbed (Table 5). This phosphorus was partly inorganic phosphate which represented about 10% of the total phosphorus content in each phytate preparation, and partly inorganic phosphate derived from the hydrolysis of phytate to inositol by an enzyme phytase present in the intestinal wall and many foods (Mellanby, 1950). The absorbed phosphorus would be expected to have a similar action on calcium excretion as the previously described neutral sodium phosphate. In four cases (3, 4, 9 and 10) the fall in urine calcium clearly exceeded the rise in fæcal calcium and in each case retention of phosphorus occurred. In the other three cases the correlation was less close. Cases 2 and 5 were spoilt by collection errors (see legends to Figs. 2 and 5) and in Case 8 the close proximity of the immediately adjacent periods 3 and 6 on sodium bicarbonate and sodium phosphate almost certainly interfered with the two periods on sodium phytate. When the phytate was stopped in Cases 3, 4 and 10, the urine calcium promptly rose to the control level, accompanied by the excretion of some of the retained phosphorus. In normal subjects McCance and Widdowson (1942) found a fall in urine calcium which was smaller than the rise in fæcal calcium, producing a negative balance of 75–175 mg./day and no change in the phosphorus balance despite 40–60% hydrolysis of the phytate. Henneman, Carrol and Albright (1956) and Henneman *et al.* (1958) found a similar response in cases of idiopathic hypercalciuria, but their cases were studied on a low calcium intake which may well have intensified the action of the phytate on intestinal absorption, producing a negative balance. They did not find the phosphorus effect we have described, either with sodium phosphate or phytate, but the effect has been observed using phytate in one case of sarcoidosis reported by these workers (Henneman *et al.*, 1956). A fall in urine calcium and phosphorus retention has been observed by Jackson and Dancaster (1959) in both sarcoidosis and idiopathic hypercalciuria.

Cellulose phosphate is an ion exchange substance with a particular affinity for divalent cations due to the steric configuration of the phosphate groups. The drug provided a unique opportunity for studying the effects of a substance which interferes with calcium absorption without the complication of a large increase of absorbed phosphorus. In all cases there was a rise in fæcal calcium, a fall in urine calcium and no significant change in the calcium balance. Such a rapid fall in urine calcium strongly suggests that these patients were already hyperabsorbers of calcium. One patient (9) had sarcoidosis, where the existence of hyperabsorption is well known. The second was a case of primary hyperparathyroidism (8). In the form of this disease where overt bone involvement apparently does not develop intestinal over-absorption could well be the source of the increased blood and urine calcium. A direct action of parathyroid hormone on intestinal absorption has been suggested by Bauer, Albright and Aub (1930) from balance studies and by Rasmussen (1959) and Dowdle, Schachter and Schenker (1960). The third patient with idiopathic hypercalciuria (5) could be representative of other patients with this disorder. The full implications of these findings must await further studies but the preliminary results illustrate the usefulness of cellulose phosphate in the investigation of such patients.

Summary

1. The results are presented of calcium, phosphorus and nitrogen balance in five patients with idiopathic hypercalciuria, three patients with primary hyperparathyroidism and one patient each with sarcoidosis and Paget's disease with hypercalciuria. Various drugs were administered orally.

2. The patients with idiopathic hypercalciuria were all in slightly negative calcium balance with a low fæcal calcium. The same was found in two of the patients with hyperparathyroidism and the patients with sarcoidosis and Paget's disease.

3. In all patients ammonium chloride increased and sodium bicarbonate significantly lowered urine calcium.

4. Neutral sodium phosphate lowered urine calcium with no significant change in fæcal calcium and hence increased calcium retention.

5. Sodium phytate produced a moderate rise in fæcal calcium and a greater fall in urine calcium with increased retention of calcium and phosphorus.

6. Cellulose phosphate produced a rise in fæcal calcium and a corresponding fall in urine calcium with no change in calcium balance and no effect due to its extra phosphorus content.

7. Cortisone increased fæcal calcium and lowered urine calcium in the patient with sarcoidosis and increased the urine calcium and slightly decreased the fæcal calcium in the cases of hyperparathyroidism and idiopathic hypercalciuria.

8. The nature of idiopathic hypercalciuria is discussed in terms of the two theories which have been put forward to explain it.

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APPENDIX

SUMMARIES OF CASE HISTORIES

Idiopathic hypercalciuria

Case 1—R.P.

A twenty-eight year old male clerk, who first passed a stone two years previously and a second just prior to admission. Physical examination was normal.

Case 2—R.D.

A thirty-six year old male engineer who had had numerous attacks of renal colic and hæmaturia for ten years. In 1958 a large stone was removed from the left kidney. He also suffered from hypertension—B.P. 210/170. Otherwise physical examination was normal.

Case 3—T.G.

A forty-five year old labourer with intermittent pain in the right loin for three years. He had passed a stone. Physical examination was normal except for an enlarged prostate gland.

Case 4—W.M.

A forty-one year old gas meter reader, who first passed a stone sixteen years previously. He had had unsuccessful exploration for a parathyroid tumour in 1956 and in 1957 a sympathetic denervation of the right kidney for persistent pain. Physical examination was normal except for tenderness in the right loin.

Case 5—A.L.

A forty-four year old chauffeur with hæmaturia and renal colic for thirteen years. He developed pyelonephritis and was found to have bilateral kidney stone, a bifid right renal pelvis and a persistent urinary infection with B. aerogenes and E. coli. Physical examination was normal.

Primary hyperparathyroidism without bone disease

Case 6—E.L.

A fifty-two year old housewife who had stones removed from the left kidney six and eight years previously. Physical examination was normal except for a blood pressure of 200/120. At the conclusion of the metabolic study an adenoma of the left inferior parathyroid gland was removed by Mr. V. H. Riddell.

Case 7—T.R.

A thirty-one year old marine engineer who had a right sided loin pain for two years. Physical examination was normal except for a blood pressure of 160/100. The serum calcium fluctuated between 10·6 and 11·2 mg./100 ml. and serum phosphorus between 3·6 and 4·0 mg./100 ml.

Plasma calcium fractionation showed a raised ionised calcium of 6·7 mg./100 ml. A strontium tracer test at Hammersmith Hospital showed Ca_F 12·1 plasma units and Ca_D 2·4 plasma units, indicating abnormally rapid bone turnover (Fraser, Harrison and Ibbertson, 1960). The phosphorus excretion index (P.E.I.) was normal (−0·013 and −0·002). At operation (Mr. D. R. Davies) all four parathyroid glands were identified and biopsied and the right upper gland and left lower gland were removed. Examination of the sections showed chief cell hyperplasia.

Case 8—K.T.

A fifty-seven year old insurance official who had a stone removed from the left kidney twenty-five years previously. He passed further stones every four to five years and had a history of gout. Physical examination was normal. The serum calcium varied from 10·4 to 12·4 mg./100 ml.

A plasma calcium fractionation was initially normal (Dr. D. Smeenk) but six months later was repeated (Dr. L. Watson) and the ionised calcium found to be unequivocally raised at 7·0 mg./100 ml. Sir R. Marnham explored the neck and removed a minute parathyroid adenoma.

Sarcoidosis with hypercalciuria

Case 9—M.H.

A forty-seven year old, coloured West Indian leather worker who came under investigation after an attack of right renal colic. Physical examination was normal. Additional investigations to those in Table I were: Mantoux 1/1,000 negative, positive at 1/100; chest X-ray showed bilateral lower zone reticulation and striation without hilar gland enlargement; liver function tests—thymol turbidity 17 units, zinc sulphate turbidity 13 units; liver biopsy showed appearances typical of sarcoidosis; iliac crest bone biopsy showed normal bone, but a sarcoid granuloma in the marrow spaces. (It was impossible to say whether the raised alkaline phosphatase was due to liver or bone involvement.)

Paget's disease with hypercalciuria

Case 10—F.B.

A fifty-three year old service station attendant who had a stone removed from the right ureter ten years previously and a year later a partial left nephrectomy. At this time the alkaline phosphatase was 10·5 King-Armstrong units, but there was radiological evidence of Paget's disease of the lumbar spine. He continued to pass stones about once a year. Physical examination was normal.

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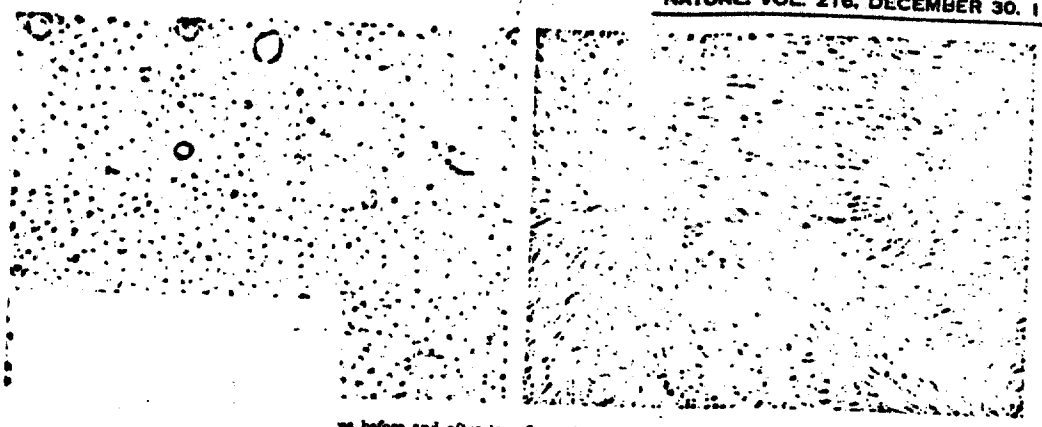

...es before and after transformation; left, normal (epithelial morphology); right, transformed (fibroblast-like morphology).

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A.P. Perris

Calcium and the Control of Mitosis in the Mammal

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INCREASING the calcium concentration in the environment of various types of isolated mammalian and insect cells hastens their progress through mitosis and also counteracts the transient mitotic inhibition following irradiation[1-6]. The same effects can be produced in the bone marrow and thymus of the whole animal by raising the blood calcium either directly, by injecting calcium salts, or indirectly through the action of parathyroid hormone[7,8]. Conversely, lowering the calcium level in the medium of isolated normal or irradiated cells reduces their rate of flow into mitosis[6]. Reducing the total calcium level in the blood by injecting the chelating compound ethylene diamine tetra-acetic acid (EDTA) or inorganic phosphate should therefore reduce the mitotic activity in normal and irradiated marrow and thymus *in vivo*. Contrary to expectations, the present study shows that these compounds in fact stimulated mitosis.

At the start of any experiment, or immediately after irradiation (cobalt-60 γ-radiation), male Sprague–Dawley rats weighing 125 g were injected intraperitoneally with 0·1 mg of colchicine/100 g body weight, and were given a second injection of this same amount 2·5 h later. The progress of cells through mitosis is thus arrested at metaphase and their rate of entry into mitosis can be determined[9]. Either EDTA or disodium hydrogen phosphate was injected intraperitoneally immediately after the first colchicine injection. EDTA (30 mg/ml.) adjusted to *p*H 7·2 with sodium hydroxide, and disodium hydrogen phosphate solution (80 mmolar) buffered to *p*H 7·2 in 5 mmolar *tris* (hydroxymethyl) aminomethane (*tris*), were given as a dose of 1·0 ml./100 g. Neither the stress of injection nor the *tris* buffer affected mitotic activity[8].

At various times after the beginning of the experiment, marrow and thymus cell suspensions were prepared as described elsewhere[9]. Samples of these suspensions were fixed in neutral formalin, stained in Delafield's haematoxylin and the percentage of cells in metaphase was then determined by examining at least a thousand cells.

Blood calcium levels were determined by removing blood samples from the abdominal aorta every 10 min after the injection of EDTA or phosphate; the concentration of total calcium was determined using the colorimetric method of Copp *et al.*[10], and the ionized calcium concentration was determined with a specific calcium ion electrode (Orion Research, Ltd., Cambridge, Massachusetts).

In the normal animal, bone marrow cells progressed into mitosis in a regular fashion (Fig. 1*A*), and by 6 h.

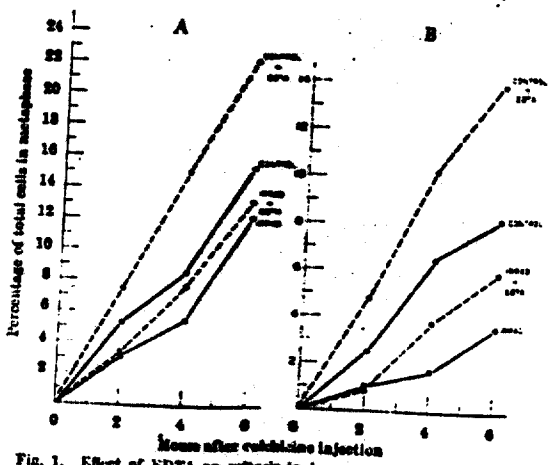

Fig. 1. Effect of EDTA on mitosis in bone marrow and thymus of normal and irradiated Sprague–Dawley rats. *A*, Bone marrow; *B*, thymus. In non-irradiated animals, the differences between the percentage of cells trapped at metaphase in normal and EDTA-treated animals were already significant by 2 h after the initial colchicine injection; $P < 0·05$ (bone marrow) and $P < 0·01$ (thymus). In irradiated (100 r.) animals, EDTA caused a significant stimulation of mitosis in the thymus 4 h after the colchicine injection, $P < 0·01$. The consistent but slight stimulation in the bone marrow was not significant, $P < 0·1$. Each point is a mean value from at least four rats.

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3·3 per cent of the cell population had collected at metaphase. EDTA stimulated mitosis; this stimulation became evident as early as 2 h after treatment, and by 4 h, 22·1 per cent of the cells had reached metaphase (Fig. 1*A*). Thymus tissue was less active mitotically than marrow and only 8·2 per cent of normal cells reached metaphase in 6 h, but EDTA treatment increased this reaction to 14·0 per cent (Fig. 1*B*). Phosphate solution also accelerated mitosis in thymus and marrow, but it was slightly less effective than EDTA (Fig. 2).

EDTA and phosphate also stimulated mitosis in the thymus and marrow of irradiated animals. During the first 2 h after irradiation and injection of either compound, there was no demonstrable effect. Between 2 and 6 h, however, a stimulation by these compounds became evident (Figs. 1 and 2).

Despite the fact that both EDTA and phosphate stimulated mitosis, they also caused a decline in the level of both total and ionized calcium during the first 10 min after injection. The total calcium level subsequently returned to normal, but never exceeded this value. The majority of this restorative calcium surge, however, consisted of ionized calcium and its level rose well above the normal value. Thus 80 min after treatment with EDTA, when the total calcium had returned to normal, as much as 93 per cent of it was ionized compared with 67 per cent in the normal animal (Figs. 3 and 4).

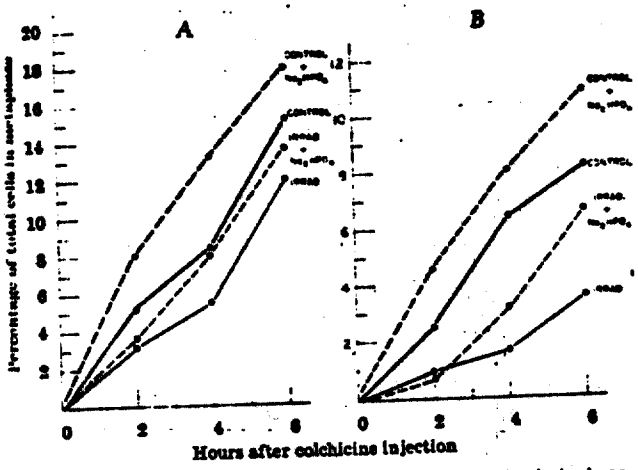

Fig. 2. Effect of disodium hydrogen phosphate on mitosis in bone marrow and thymus of normal and irradiated Sprague–Dawley rats. *A*, Bone marrow; *B*, thymus. In non-irradiated animals, the differences between the percentage of cells trapped at metaphase in normal and phosphate-treated animals were already significant by 2 h after the initial colchicine injection in both marrow and thymus ($P < 0·01$). In irradiated (100 r.) animals phosphate produced a stimulation of mitosis by 4 h in both tissues, $P < 0·05$ (marrow), $P < 0·02$ (thymus). Each point is a mean value from at least four rats.

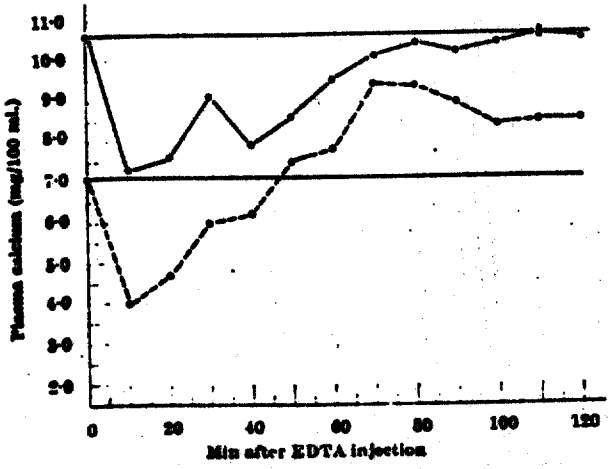

Fig. 3. Effect of injection of EDTA on total (——) and ionized (– – – –) plasma calcium levels. Each point is a mean value from four rats.

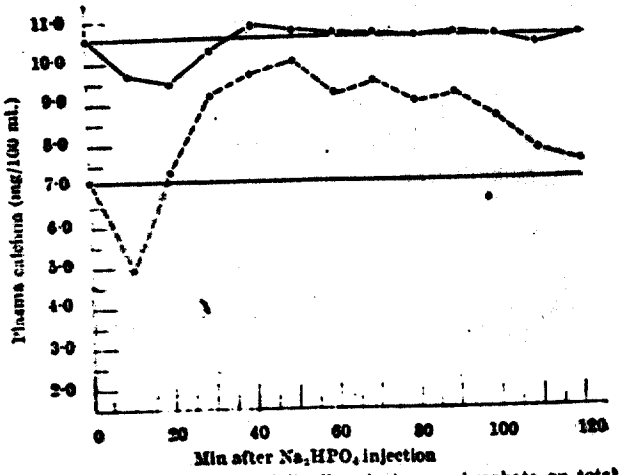

Fig. 4. Effect of injection of disodium hydrogen phosphate on total (——) and ionized (– – – –) plasma calcium levels. Each point is a mean value from three rats.

The unexpected stimulation of mitosis produced by EDTA and phosphate can now be attributed to the abnormally elevated ionized calcium levels which follow the initial lowering of this level induced by these compounds. Mitotic activity can obviously be affected by merely changing the level of ionized, in contrast to bound, calcium in the cellular environment. Plasma calcium levels are controlled by two antagonistic hormones: of these, parathyroid hormone causes a surge of ionized calcium which restores total calcium levels to normal after a sudden depression as in the present study, and calcitonin acts to depress abnormally high levels[11–13]. The present results imply therefore that any changes in the balance of these hormones will probably be accompanied by changes in the mitotic activity of the lymphatic and haemopoietic tissues of the animal.

Regardless of the possible mechanisms by which calcium stimulates division in the mitotically competent cell[1–3,6,14], in any future consideration of mitotic stimulation and control in the mammal, the role of a calcium and the interplay of the two principal hormones which affect its level cannot be neglected.

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VARIATIONS PONDÉRALES DES PARATHYROÏDES EXTERNES DU LAPIN ET DU CHIEN SOUS L'INFLUENCE D'INJECTIONS PARENTÉRALES RÉPÉTÉES DE PHOSPHATE DE SODIUM ET DU GLUCONATE DE CALCIUM,

par M. PIERRE, P. DE BOISSEZON et CH. LOMBARD.

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On sait le rôle important que jouent les parathyroïdes dans la régulation du métabolisme phospho-calcique dont les modifications ont été étudiées aussi bien sur l'animal parathyroïdectomisé qu'en état d'hyperparathyroïdie expérimentale. Un certain nombre de travaux plaident en faveur d'une sorte de régulation automatique de la sécrétion parathyroïdienne par les modifications de la composition minérale des humeurs. Luce (1923) chez des rats, Marine (1913), Oberling et Guérin (1934) chez des poules soumis à un régime pauvre en calcium ont trouvé une augmentation du volume des parathyroïdes par hyperplasie cellulaire.

Drake, Albright et Castleman (1937) ont également signalé de l'hyperplasie de ces glandules consécutivement à des injections parentérales de phosphate de soude à des lapins.

Afin d'apporter une contribution à cette étude, nous avons entrepris une série d'expériences portant sur 30 lapins et 15 chiens. Les animaux étaient divisés en trois lots égaux de 10 lapins et 5 chiens. Les sujets du premier groupe conservés comme témoins, furent simplement nourris de la même façon que leurs congénères. Les animaux du deuxième lot reçurent par voie parentérale (intrapéritonéale chez les lapins, intraveineuse chez les chiens) des doses progressivement croissantes de phosphate de sodium à $pH = 7,2$. Quant aux individus du troisième groupe, ils furent soumis à des injections quotidiennes de gluconate de calcium dans les mêmes conditions. Le nombre des injections s'est échelonné suivant les sujets de trois semaines à deux mois.

Comme il existe d'importantes variations pondérales individuelles des parathyroïdes externes, nous avons rapporté le poids du tissu parathyroïdien et chacun des groupes à 1 kgr. d'animal. Les chiffres que nous donnons sont, par conséquent, des chiffres moyens, mais nous les considérons comme suffisamment démonstratifs pour mériter d'être signalés.

Espèce animale	Lots d'animaux	Mgr. de tissu parathyroïdien pour 1 kgr. de poids vif
Lapins.	Témoins	2,83
	Calcium	2,39
	Phosphore	3,94
Chiens.	Témoins	1,22
	Calcium	0,60
	Phosphore	1,27

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A la lecture de ce tableau, nous voyons que les lapins paraissent réagir plus intensément à l'hyperphosphatémie qu'à l'hypercalcémie alors que pour les chiens les effets sont inversés. Nous voyons de plus, que tout se passe comme si les animaux traités par des injections répétées de phosphate de sodium réagissaient par de l'hypertrophie parathyroïdienne tandis que ceux qui reçoivent le gluconate de calcium répondent par de l'hypotrophie.

Ces réactions parathyroïdiennes correspondent-elles à des modifications de l'activité physiologique glandulaire ? Les expériences réalisées ne nous permettent pas de répondre à cette question. Des recherches en cours nous feront peut-être connaître la nature des remaniements histologiques responsables des variations pondérales enregistrées.

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DIMOSTRAZIONE DELL'EFFICACIA DEL FOSFATO BIAMMONICO QUALE FONTE ALIMENTARE DI FOSFORO

*Ricerche condotte con fosfato marcato con ^{32}P **

Ormai da molti decenni ricercatori di tutti i paesi hanno richiamato l'attenzione sulla frequenza di troppo bassi tenori in fosforo dei foraggi e sui correlati stati nutrizionali degli animali, ruminanti in particolare. Anche nel nostro paese il problema delle carenze di fosforo, specie sui bovini, è di estrema attualità e non sembra inutile ricordare che, contrariamente ad opinioni ancora molto diffuse tra gli allevatori, simili carenze non si risolvono solo a carico dello scheletro.

Ben il 20 % del fosforo totale presente nell'organismo animale non fa parte dello scheletro e ciò in dipendenza delle molteplici funzioni che questo elemento svolge accanto a quella plastica; basti pensare che l'acido fosforico, che entra in gioco nell'attivazione dei glucidi, è costituente del Coenzima A, indispensabile per l'attivazione degli acidi grassi: che i processi di fosforilazione hanno importanza anche per l'assorbimento intestinale dei glucidi e dei lipidi e per il riassorbimento del glucosio a livello dei tubuli renali: che le lecitine intervengono nel trasporto dei lipidi e degli acidi nucleinici, nella biosintesi delle proteine specifiche dei tessuti. E ancora: l'acido fosforico entra nella costituzione dei diversi coenzimi di fondamentale importanza biologica ed interviene nei processi di accumulo, trasferimento e cessione di energia.

Il fabbisogno in fosforo è strettamente legato a momenti fisiologici della vita dell'animale: basti ad esempio pensare che con 1 litro di latte vengono eliminati da g 0,53 a g 1,18 di fosforo e che negli ultimi due

* Lavoro eseguito con il contributo del C.N.R.

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mesi di gravidanza della vacca il passaggio di fosforo attraverso la placenta può essere di circa g 5 al giorno (HEMINGWAY, 1967).

In pratica il continuo miglioramento dell'attitudine produttiva degli animali di interesse zootecnico ha reso sempre più frequente la necessità di apporti supplementari di fosforo: innaturali produzioni esigono ovviamente innaturali apporti di principi alimentari essenziali.

Va per altro precisato che in molte zone, caratterizzate da terreni poveri in fosforo, un'integrazione alimentare con lo stesso elemento si impone anche per sostenere livelli produttivi molto bassi.

O'MOORE (1960) ha descritto gli effetti sui bovini dei foraggi con tenori via via più bassi di fosforo. Foraggi con lo 0,26 % di fosforo si dimostrarono idonei a coprire il fabbisogno di mantenimento e produzione di 13,5 litri di latte al giorno; mentre con lo 0,17 si manifestarono segni di afosforosi sia nelle vacche che nelle manze (irregolarità di ciclo estrale, ritardo della crescenza, minor produzione di latte); al di sotto dello 0,15 % si notarono danni evidenti allo scheletro.

HEMINGWAY (1963) indusse in agnelli di due mesi a dieta a basso tenore in fosforo (inferiore allo 0,10 %) gravi sintomi di rachitismo e deformazione degli arti.

DUNCAN (1958) a seguito di una lunga sperimentazione ha potuto concludere che un insufficiente apporto alimentare di fosforo si ripercuote sul tenore in fosforo del sangue assai più prontamente di quanto non accada per la calcemia in difetto di calcio.

La normale concentrazione di fosforo inorganico nel sangue è di 4-8 mg per 100 ml ma si possono riscontrare anche valori più alti. In condizioni di carenza lo stesso tasso ematico può scendere a 1-2 mg per 100 ml.

Ad esempio O'MOORE (1950) in bovini mantenuti per due mesi con foraggio a tenore in fosforo inferiore allo 0,15 % ha riscontrato una fosfatemia pari a 2 mg per 100 ml.

Va ancora ricordato che non di rado il foraggio non sa coprire il fabbisogno in fosforo, non tanto in funzione di un difetto in questo elemento, quanto in dipendenza di un troppo elevato rapporto calcio/fosforo (> 2:1), il che riduce l'assorbimento di fosforo in sede intestinale. È questa un'evenienza che più frequentemente si verifica con foraggi costituiti da leguminose e che contribuisce a giustificare talune negative influenze che le razioni a base di leguminose possono esplicare a carico dell'apparato riproduttore.

Nell'usuale razionamento dei nostri animali l'apporto di fosforo è di solito assicurato da un supplemento di fosfato bicalcico (18 % di

fosforo e 23 % di calcio) meno frequentemente da un apporto di fosfato bisodico (11,56 % di fosforo).

RUSSEL e coll. nel 1961 preconizzarono la possibilità di usare il fosfato biammonico quale fonte di azoto e di fosforo per i ruminanti.

Nell'anno successivo COWMANN e coll. con prove pratiche su vitelloni Hereford, ritennero di poter concludere che nei gruppi alimentati con razioni a basso tenore in proteine e in fosforo, integrati con fosfato biammonico e monoammonico, gli incrementi ponderali ed il tenore in fosforo del sangue non erano dissimili da quelli evidenziati nei gruppi alimentati con razioni ad apporto proteico affidato alla soia ed arricchiti in fosforo con fosforiti defluorizzate. Peraltro le modalità con le quali queste esperienze pratiche sono state condotte non potevano autorizzare sicure conclusioni.

Nell'ultimo lustro con una larga sperimentazione condotta nei nostri istituti (1966 *a*, 1966 *b*, 1967, 1968, 1968 *b*) impiegando fosfato biammonico marcato con ^{15}N potevamo fornire una diretta ed inequivocabile dimostrazione della capacità dei microrganismi ruminali di utilizzare l'azoto del fosfato biammonico ai fini della sintesi delle loro proteine ed altresì evidenziare (PIANA-PIVA, 1968 *b*) una modalità pratica per valorizzare questa ed altre fonti di azoto non protidico.

Recentemente ci siamo anche occupati di controllare, sempre in via diretta utilizzando fosfato biammonico marcato con ^{32}P, l'utilizzazione del fosforo del fosfato biammonico in confronto a quello del fosforo del fosfato bicalcico, certo l'integratore fosforico più impegnato in sede zootecnica.

Sia il fosfato biammonico che il fosfato bicalcico marcati sono stati preparati direttamente in laboratorio a partire da acido fosforico marcato con 1 mc di ^{32}P. Il controllo del tenore in ^{32}P dei due prodotti così ottenuti è stato effettuato con un contatore a bicchiere della 20th Century Electronics mod. 116 abbinato ad uno Scaler della S.E.L.O.

Su una pecora in lattazione del peso di circa kg 40 sono state condotte in due periodi successivi le seguenti prove:

1) alimentazione per 45 giorni — cominciando da 25 giorni prima del parto — con una razione costituita per il 50 % da fieno scadente di prato stabile e per il 50 % da una miscela di concentrati contenente il 3 % di fosfato biammonico (miscela concentrati: mais 61,5 % - amido 27,5 % - sale pastorizio 1,0 % - siero latte 5,0 % - fosfato biammonico 3,0 % - carbonato di calcio 2,0 %. Integrazione vitaminica:

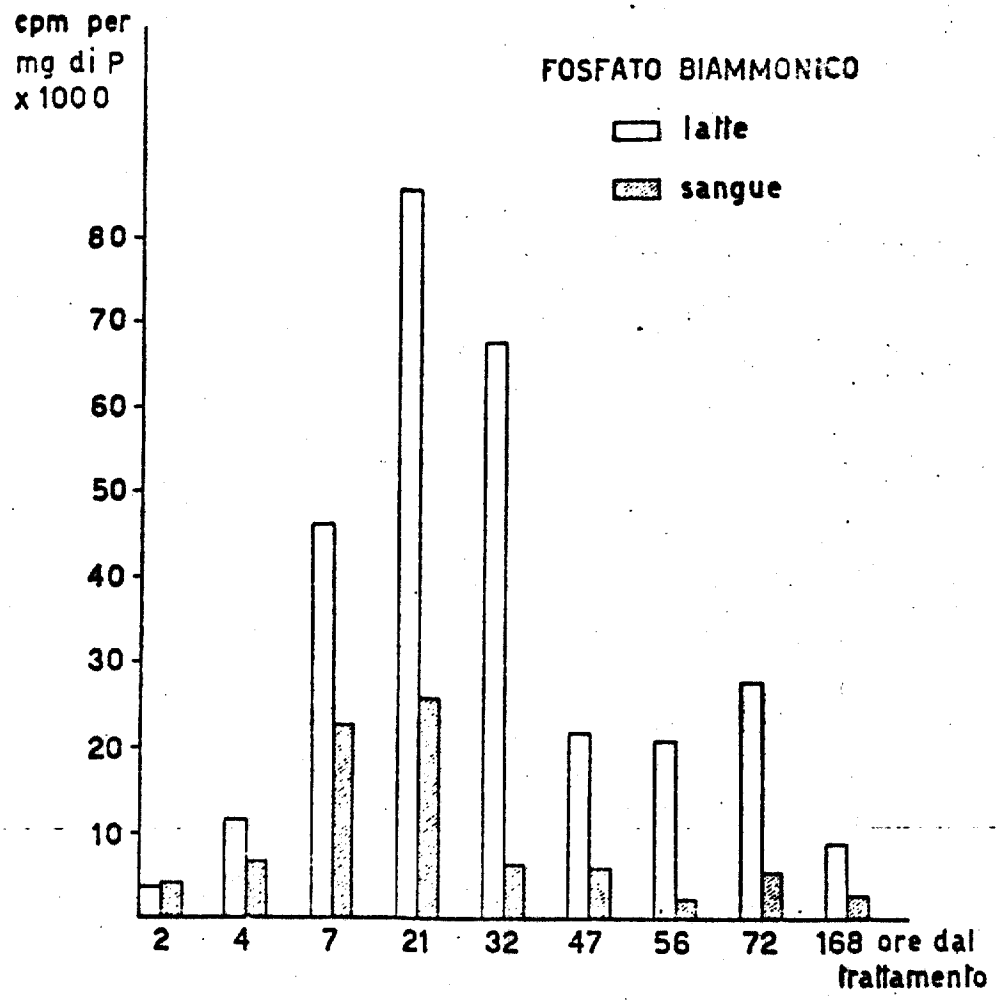
vitamina A 10.000 U.I./kg - vitamina D_3 5.000 U.I./kg). Dopo questo periodo di assuefazione, sostituzione del fosfato biammonico della miscela alimentare con una somministrazione direttamente in rumine per sonda di g 9 di fosfato biammonico marcato in soluzione acquosa con un'attività specifica di 614.654 cpm per mg di fosforo. Prima della somministrazione del prodotto marcato l'animale è stato sottoposto ad una mungitura a fondo; in seguito ed a partire dalla 2ª ora dopo il trattamento le mungiture sono state ripetute a diversi intervalli di tempo come indicato della tabella I: contemporaneamente sono stati eseguiti prelievi di sangue dalla giugulare. Su tutti i campioni prelevati abbiamo determinato sia il fosforo totale previa digestione della sostanza organica con acido solforico ed usando come reagente specifico del fosforo il vanadomolibdato di ammonio (1965), sia la sua attività specifica sempre dopo mineralizzazione con acido solforico in presenza di solfato di potassio e solfato di rame come catalizzatori fino a soluzione limpida. Dopo 168 ore dalla somministrazione del prodotto marcato abbiamo sospeso i prelievi in quanto il controllo dei cpm del sangue aveva dimostrato bassi livelli.

I risultati ottenuti sono illustrati dalle tabelle I e II e dal grafico n. 1:

TABELLA I

Tenore in ^{32}P del fosforo ematico della pecora dopo diversi intervalli di tempo dalla somministrazione una tantum *di g 9 di fosfato biammonico marcato con ^{32}P (a cpm 614.654 per mg di P)*

Ore dalla somministrazione	P totale mg/100 ml	cpm per mg di P
Prima del trattamento	38,70	—
Dopo 2 ore	38,90	3.968
» 4 »	36,00	6.659
» 7 »	38,14	22.688
» 21 »	40,00	26.074
» 32 »	41,30	6.373
» 47 »	46,70	5.861
» 56 »	39,20	2.036
» 74 »	38,90	5.118
» 168 »	39,80	2.940


cpm per
mg di P
x 1000
FOSFATO BIAMMONICO
latte
sangue
80
70
60
50
40
30
20
10
2
4
7
21
32
47
56
72
168 ore dal
trattamento

GRAF. n. 1

2) nella stessa pecora, dopo un intervallo di tempo di alcune settimane è stata ripetuta l'esperienza seguendo le stesse modalità, ma sostituendo, sia nel periodo di assuefazione che nel periodo sperimentale vero e proprio, al fosfato biammonico il fosfato bicalcico in dosi equivalenti per apporto di fosforo (miscela concentrati: mais 61,5 % - amido 28,9 % - sale pastorizio 1,0 % - siero latte 5,0 % - fosfato bicalcico 3,2 % - carbonato di calcio 0,4 % - integrazione vitaminica: vitamina A 10.000 U.I./kg - vitamina D_3 5.000 U.I./kg).

TABELLA II

Tenore in ^{32}P del fosforo del latte di pecora dopo diversi intervalli di tempo dalla somministrazione una tantum *di g 9 di fosfato biammonico marcato con ^{32}P (a cpm 614.654 per mg di P)*

Ore dalla somministrazione	P totale mg/100 ml	cpm per mg di P
Prima del trattamento	116	—
Dopo 2 ore	124	3.526
» 4 »	131	10.155
» 7 »	118	46.336
» 21 »	108	85.886
» 32 »	121	67.639
» 47 »	125	21.678
» 56 »	115	20.735
» 74 »	122	27.778
» 168 »	135	8.809

Il fosfato bicalcico impiegato aveva un'attività specifica uguale a quella del fosfato biammonico impiegato nella precedente esperienza.

Nelle tabelle III e IV e dal grafico n. 2 sono illustrati i risultati raggiunti in questa seconda esperienza.

Il confronto dei dati raccolti nelle due esperienze mette in evidenza che le variazioni del tenore in ^{32}P nel sangue, dopo somministrazione di fosfato biammonico o fosfato bicalcico marcati hanno un andamento nel tempo pressoché sovrapponibile (almeno fino alla 168ª ora) il che deporrebbe per un assorbimento del tutto analogo per i due sali.

TABELLA III

Tenore in ^{32}P del fosforo ematico della pecora prima e a diversi intervalli di tempo dalla somministrazione una tantum *di g 9 di fosfato bicalcico marcato con ^{32}P*

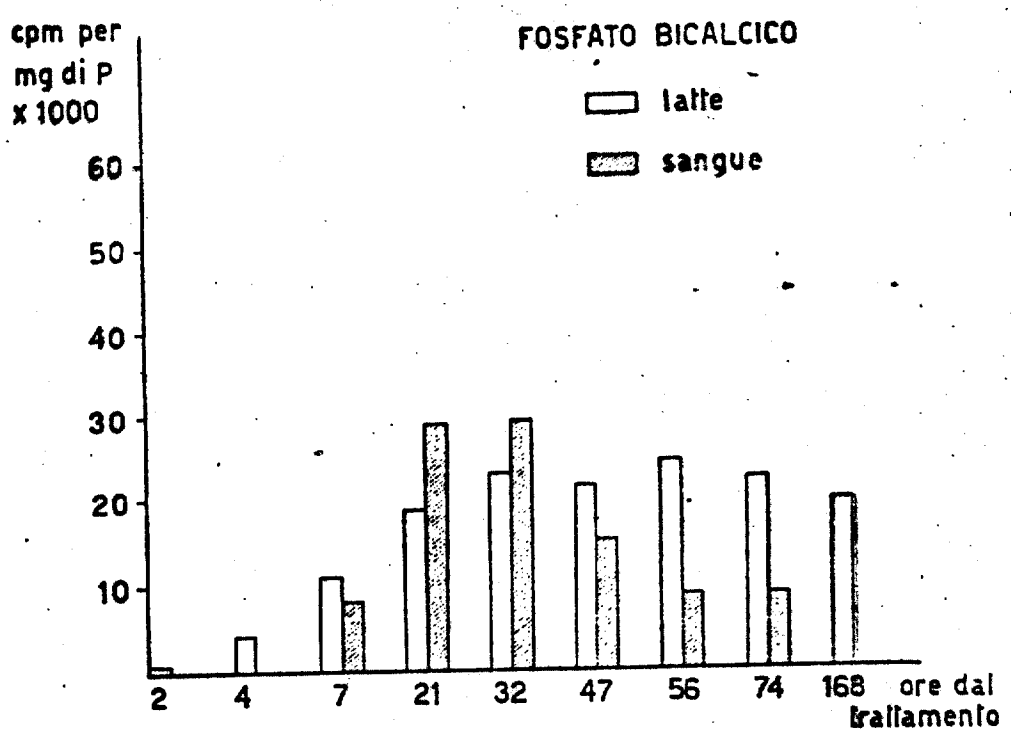
Ore dalla somministrazione	P totale mg/100 ml	cpm per mg di P
Prima del trattamento	39,10	—
Dopo 2 ore	39,20	—*
» 4 »	39,57	—*
» 7 »	35,60	7.994
» 21 »	38,90	29.010
» 32 »	39,87	29.336
» 47 »	38,96	15.040
» 56 »	35,80	8.694
» 74 »	36,95	8.642
» 168 »	43,00	—*

* Su questi campioni non è stato possibile eseguire l'analisi.

TABELLA IV

Tenore in ^{32}P del latte di pecora prima e a diversi intervalli di tempo dalla somministrazione una tantum *di g 9 di fosfato bicalcico marcato con ^{32}P*

Ore dalla somministrazione	P totale mg/100 ml	cpm per mg di P
Prima del trattamento	114	—
Dopo 2 ore	104	650
» 4 »	111	4.015
» 7 »	114	11.112
» 21 »	117	18.751
» 32 »	114	22.693
» 47 »	122	21.545
» 56 »	108	24.438
» 74 »	125	22.402
» 168 »	124	19.663



GRAF. n. 2

Diversamente si presentano i due grafici relativi all'andamento dei tenori in ^{32}P del latte dopo la somministrazione dell'uno e dell'altro fosfato, nel senso che la eliminazione del P marcato attraverso la mammella appare assai più rapida ed intensa di quanto non accada per il ^{32}P somministrato sotto forma di fosfato bicalcico.

La constatazione sembrerebbe indicare che nel fosfato biammonico vada identificata una fonte di fosforo particolarmente adatta a soddisfare il fabbisogno in questo elemento della ghiandola mammaria.

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RIASSUNTO

Gli autori prendono in esame la possibilità di utilizzare il fosfato biammonico come fonte di fosforo per i ruminanti. La ricerca è stata condotta con l'impiego di fosfato biammonico marcato con ^{32}P e gli autori giungono alla conclusione che i ruminanti sono in grado di utilizzare il fosforo del fosfato biammonico per la sintesi del latte.

RÉSUMÉ

Les Auteurs examinent la possibilité d'utiliser le biphosphate d'ammonium comme source de phosphore pour les ruminants. Les recherches correspondantes ont été effectuées avec emploi de biphosphate d'ammonium marqué par le P^{32} et les Auteurs parviennent à la conclusion que les ruminants sont en mesure d'utiliser le phosphore du biphosphate d'ammonium pour la synthèse du lait.

SUMMARY

The authors investigated the alimentary role of diammonium phosphate in ruminants sheep as source of P. For this study diammonium phosphate labelled with ^{32}P was used. The authors conclude that the ruminant is able to use the phosphorus of diammonium phosphate for the synthesis of milk.

Amer. J. Pathol. 17(4): 590 (1941)

792

(Dr. Stewart) How long had the stock been separated from the parent strain?

(Dr. Schenken) Since 1937.

EFFECTS OF LONG-CONTINUED INGESTION OF SODIUM PHOSPHATE UPON THE PARATHYROIDS, KIDNEYS AND BONES OF MATURE RATS. John A. Saxton, Jr., and (by invitation) Gordon H. Ellis, New York City and Ithaca, N.Y.

Abstract. Mature male rats were divided into two lots. One lot was fed an adequate diet containing 8 per cent of sodium metaphosphate. The other lot received a diet containing about the same quantity of sodium orthophosphate. These diets were continued for 7 months, or until the animals succumbed. X-ray examinations at intervals during life disclosed a gradual decalcification of bones and deposition of calcium in the kidneys. Urinary excretion of inorganic phosphorus was increased. Gross examination showed enlargement of the parathyroids from two to eight times that of the controls. The long bones appeared slightly thickened, but in some instances were more fragile than normal. Microscopic study showed hypertrophy and hyperplasia of parathyroid cells. Calcium deposits were present in the tubules of the kidneys, chiefly in the medulla. There were widespread metastatic calcium deposits in numerous organs. The long bones showed diffuse endosteal and periosteal new-bone formation with focal areas of osteoclasis and fibrosis. These observations suggest that some instances of human disease in which there are lesions of the osseous system and parathyroid hyperplasia may be a consequence of excessive ingestion of phosphates.

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[illegible] the complex, which should be called the neuro-insular complex, was found.

The structure of the complex is not uniform; rather it is characterized by a great variability as regards the proportion of epithelial and ganglion cells. The epithelial cells of the complex are insular cells. From the histophysiological point of view the neuro-insular complex of the pancreas might be considered as a typical neurocrine organ, being a part of the intrinsic nervous system of the gland. It might be an important organ in the autonomic regulation of insulinic secretion.

Total cross sections of the pancreases taken from persons who had died in diabetic coma were carefully examined by serial sections. In 1 case only two

 Proc. Soc. Exp. Biol. Med. 52: 274-5 (1943)

14118

Effec. of Oil of Peppermint, Dehydrocholic Acid and Disodium Phosphate on Gastric Emptying in Dogs.

873

J. CLIFFORD STICKNEY. (Introduced by Edward J. Van Liere.)

From the Department of Physiology, School of Medicine, West Virginia University, Morgantown, W.Va.

The results reported here were obtained in an endeavor to find a simple substance which would reduce to normal the increased gastric emptying time produced by anoxia. Oil of peppermint,[1] dehydrocholic acid[2] and disodium phosphate[3] were chosen, because they have been reported to decrease gastric emptying time in human beings and the first named agent in dogs as well. In addition they have little or no complicating side effects.

Normal gastric emptying time was determined fluoroscopically on 4 dogs following a meal of 10 g ground dried bread, 20 g ground lean beef and 50 cc skim milk to which 15 g barium sulfate were added. From 6 to 11 control determinations were made on each dog to establish the mean. In the case of dogs 2, 3 and 4 the control and experimental values were obtained with the dogs in a low-pressure chamber at normal atmospheric pressure when the experiments with oil of peppermint were undertaken. For the other experiments the dogs were kept in their accustomed quarters. The dehydrocholic acid and the disodium phosphate were mixed with the test meal while the oil of peppermint was given orally in a capsule immediately before the test meal.

The dogs weighed from 8.6 to 9.8 kg. The effect of oil of peppermint was tested at three dosage levels: 0.1, 0.2 and 1.0 cc. Dog 4 vomited on two occasions with the 1.0 cc dose, so it was concluded that no larger amount could profitably be given. The amount of dehydrocholic acid administered was 0.5 g. Disodium phosphate was tested at the 0.5 and 1.0 g dosage levels. In every case with one exception four or more trials were made on each drug at each dosage level.

The results are given in the accompanying tables along with the calculation of Fisher's "p"[4] to determine the significance of the differences. It will be noticed that with 2 exceptions gastric emptying time was increased in every case when the groups are taken as a whole, but with certain dogs there was a significant increase for certain dosage levels of the drugs. Dog 1, which was also given oil of peppermint at the 0.2 cc dosage level, had a significant increase in gastric emptying time after 0.1 cc and 0.2 cc oil of peppermint.

TABLE I.
Effect of 0.1 and 1.0 cc Oil of Peppermint on Gastric Emptying Time.

Dog No.	Control, hr	After 0.1 cc oil of peppermint, hr	Difference, hr	Fisher's "p"	After 1.0 cc oil of peppermint, hr	Difference, hr	Fisher's "p"
1	5.50	6.30	+.80*	<.001	5.85	+ .35*	>.05
2	5.16	5.81	+.66	>.10	5.48	+ .32	>.20
3	5.78	6.75	+.97	>.10	7.06	+1.27*	<.01
4	4.83				4.94	+ .11	>.20
Avg	5.48†	6.29	+.81	>.05	5.83	+ .51	>.20

* Significant when "p" is .05 or less.
† Dog 4 not included.

[1] Sapoznik, H. I., Arens, R. A., Meyer, J., and Neuhoes, H., *J. Am. Med. Assn.*, 1935, 104, 1792.

[2] Van Liere, E. J., and Northup, D. W., *Am. J. Dig. Dis.*, 1941, 8, 26.

[3] Van Liere, E. J., and Sleeth, C. K., *Am. J. Dig. Dis.*, 1940, 7, 118.

[4] Fisher, R. A., *Statistical Methods for Research Workers*, fourth edition, Oliver and Boyd, London, 1932.

TABLE II.
Effect of 0.5 and 1.0 g Disodium Phosphate on Gastric Emptying Time.

Dog No.	Control, hr	After 0.5 g disodium phosphate, hr	Difference, hr	Fisher's "p"	After 1.0 g disodium phosphate, hr	Difference, hr	Fisher's "p"
1	5.50	5.79	+.29	>.20	5.80	+.30	>.20
2	5.32	5.85	+.53*	>.10	5.10	—.22	>.20
4	4.94	5.30	+.36	>.10	5.62	+.69*	>.05
Avg	5.25	5.64	+.39	>.20	5.51	+.26	>.20

* Significant when "p" is .05 or less.

TABLE III.
Effect of 0.5 g Dehydrocholic Acid on Gastric Emptying Time.

Dog No.	Control, hr	After dehydrocholic acid, hr	Difference, hr	Fisher's "p"
2	5.32	5.62	+ .31	>.20
3	5.40	5.00	— .40	>.20
4	4.94	6.06	+1.12*	<.01
Avg	5.22	5.56	+ .34	>.20

* Significant when "p" is .05 or less.

In the case of oil of peppermint, the results confirm the work of Van Liere and Northup,[5] who found no change in gastric emptying time in human subjects. The lack of confirmation of the work of Sapoznik *et al.*[1] may be attributed to their use of a different method to determine gastric emptying in their dogs and also to their use of very large doses of oil of peppermint. In regard to dehydrocholic acid and disodium phosphate it is difficult to explain why the dogs reacted differently than did the human subjects of Van Liere and his coworkers.[2,3]

Conclusions. The gastric emptying time of dogs has not been found to be significantly changed on the whole by the administration of oil of peppermint, dehydrocholic acid or disodium phosphate at the dosage levels used.

[5] Van Liere, E. J., and Northup, D. W., *J. Pharm. and Exp. Ther.*, 1942, 76, 39.

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Can. J. Physiol. Pharmacol. 45(6): 957-64 (1967)

920

EFFECT OF SODIUM PYROPHOSPHATE ON EXPERIMENTAL SOFT-TISSUE CALCIFICATION AND HYPERCALCEMIA[1]

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B. TUCHWEBER AND G. GABBIANI.—Effect of sodium pyrophosphate on experimental soft-tissue calcification and hypercalcemia. Can. J. Physiol. Pharmacol. 45, 957 (1967).

Sodium pyrophosphate inhibited the cutaneous calcification induced by various metallic compounds in normal rats and in those treated with dihydrotachysterol (DHT). It also caused a transient decrease in total serum calcium in normal rats and rats treated with calcium acetate, DHT, and parathyroid extract.

Le pyrophosphate de sodium a inhibé la calcification cutanée produite par divers composés métalliques chez les rats normaux et chez ceux traités à la dihydrotachystérine (DHT). Il a causé également une diminution temporaire de la quantité totale de calcium dans le sang chez les rats normaux et chez ceux traités à l'acétate de calcium, à la DHT et à l'extrait parathyroïdien.

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Introduction

Previous studies have shown that condensed phosphates inhibit calcium salt precipitation in vitro (1) and that inorganic pyrophosphate is normally present in both plasma (2) and urine (3). Hence it was suggested that the latter compound may be an important physiologic regulator of calcification (3). It has also been reported that sodium pyrophosphate and long-chain polyphosphates inhibit both the deposition of calcium salts and the formation of a calcifiable matrix in the aorta of rats treated with large doses of vitamin D (4, 5). Moreover, in rats pretreated with dihydrotachysterol (DHT), polyphosphates prevent cutaneous calciphylaxis at the site of epilation (11).

Phosphates play an important role in the mechanism of experimental soft-tissue calcification (6–9). For example, in rats injected intravenously with lead acetate, orthophosphate increases the deposition of calcium (calcergy) at sites treated with a histamine liberator, whereas sodium pyrophosphate inhibits it (8). Sodium pyrophosphate also prevents the topical calcification produced in skin areas treated with the chlorides of rare earth metals (10).

In the experiments to be described here, we have tested the action of sodium pyrophosphate on cutaneous calcification induced by various metallic compounds in normal and DHT-pretreated animals.

In addition, we have investigated the influence of sodium pyrophosphate on the level of calcium and phosphorus in the blood and urine of rats intoxicated with calcium acetate, DHT, and parathyroid extract.

Methods

Two hundred and fifty-five female Sprague–Dawley rats from the Robidoux Farm, with a mean initial body weight of either 100 g (range 90–110 g) or

[1]This work was supported by the Medical Research Council of Canada and the U.S. Army Medical and Research Development Command (Contract No. DA-49-193-MD-2039).

Phos.

200 g (range 190–200 g), were divided into 19 groups as indicated in the tables. The following agents were administered: lead chloride ($PbCl_2$, Fisher Scientific Co., Fair Lawn, N.J., U.S.A.); holmium chloride ($HoCl_3$, K & K Laboratories, N.Y., U.S.A.); chromous chloride ($CrCl_2$, Fisher Scientific Co.); ferrous chloride ($FeCl_2 \cdot 4H_2O$, Fisher Scientific Co.); sodium pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$, J. T. Baker Chemical Co., Phillisburg, N.J., U.S.A.); sodium orthophosphate (Na_2HPO_4, Fisher Scientific Co.); calcium acetate ($Ca(CH_3 \cdot COO)_2$, Fisher Scientific Co.); dihydrotachysterol (Calcamin®, Dr. A. Wander, S.A., Bern, Switzerland); parathyroid extract (Para-thormone®, Eli Lilly Company, Indianapolis, U.S.A.).

For the sake of clarity, details of treatment are described with the results.

Blood was withdrawn by aorta puncture under ether anesthesia.

During metabolic studies, rats were allowed free access to food and water and individually housed in cages specially designed to separate urine from feces. Urine was collected in HCl at 90 min and 24 h after sodium pyrophosphate administration. In each sample of urine and blood, the concentration of calcium (12) and phosphorus (14) was determined.

The rats were maintained exclusively on Purina laboratory chow (Purina Co. of Canada) and tap water. At autopsy, the diameter of the calcified wheal produced by topical treatment was measured in millimeters and the intensity of the internal lesions gauged in terms of an arbitrary scale of 0–3 (0 = no lesion, 1 = just detectable, 2 = moderate, 3 = most intense lesions). The means with their standard errors are listed in the tables. Moreover, injection sites of lead and holmium chlorides (given alone or with sodium pyrophosphate) were analyzed for calcium (13) and phosphorus (14) content after overnight incineration at 600 °C. On one portion of fresh skin samples (dehydrated at room temperature) X-ray diffraction patterns were obtained with a Debye–Scherrer camera.

For histologic purposes, soft tissues were fixed in alcohol–formol (4 parts of absolute alcohol and 1 part of 10% neutral Formalin), embedded in paraffin, and stained by means of the von Kóssa technique. Bones were fixed in neutral Formalin, decalcified in formic acid, and stained with the PAS procedure.

Results

Effect of Sodium Pyrophosphate on Calciphylaxis

Four groups of 10 rats each (100 g body weight) were given DHT (1 mg in 0.5 ml of corn oil by stomach tube) once on the first day of the experiment. Ferrous chloride (hydrated) and chromous chloride (anhydrous) were injected subcutaneously on the back at the dose of 150 μg (of the salts) in 0.2 ml of distilled water (groups 1 and 3) and in 0.1 ml of water (groups 2 and 4), on the second day of the experiment. In addition, rats of groups 2 and 4 received sodium pyrophosphate in a dose equivalent to 0.21 mg of inorganic phosphorus in 0.1 ml distilled water subcutaneously just before and at the same site as ferrous and chromous chlorides. The experiment was terminated 6 days after DHT administration.

TABLE I

Effect of sodium pyrophosphate on cutaneous calciphylaxis

Group	Treatment*	Skin calcification (mm)
1	$FeCl_2$	18±0.15
2	$FeCl_2$ + pyrophosphate	0.4±0.4
3	$CrCl_2$	17±0.06
4	$CrCl_2$ + pyrophosphate	0.2±0.2

*All rats received DHT as indicated in the text.
NOTE: The values are averages ± standard errors.

TABLE II

Effect of sodium pyrophosphate on calcergy

Group	Treatment	Skin calcification (mm)	Calcium content (% dry weight)	Phosphorus content (% dry weight)
1	None	0	0.18±0.06	0.32±0.03
2	$PbCl_2$	14±0.3	6.3±1.0	3.5±0.4
3	$PbCl_2$ + pyrophosphate	0	1.9±0.7	1.7±0.2
4	$HoCl_3$	10±0.4	6.0±0.9	4.3±0.6
5	$HoCl_3$ + pyrophosphate	0	1.7±0.9	2.5±0.4

NOTE: The values are averages ± standard errors.

As seen in Table I, the chlorides of iron and chromium produced calcified wheals of similar sizes; these lesions were prevented by sodium pyrophosphate. On histologic examination, the macroscopic readings were confirmed.

Effect of Sodium Pyrophosphate on Calcergy

Fifty rats (100 g body weight) were divided into five equal groups and treated as shown in Table II. Lead (group 2) and holmium (group 3) chlorides were injected at the dose of 200 μg (of the salts) subcutaneously between the shoulders in 0.2 ml of distilled water and in the sacral region in 0.1 ml of water. At these sites and just before the administration of the chlorides, sodium pyrophosphate was given in a dose equivalent to 0.14 mg of inorganic phosphorus in 0.1 ml of water. The experiment was terminated 6 days later.

As shown in Table II, both lead and holmium chloride produced a topical calcified wheal, which on macroscopic examination was quite obvious. Histologically, calcium salts were found to be deposited mostly in contact with collagen fibers and we noted a moderate degree of fibrous reaction with giant cell formation (10). Sodium pyrophosphate inhibited the calcium salt precipitation induced by the metallic chlorides.

Compared with normal values, calcium and phosphorus concentrations were significantly increased ($P < 0.001$) in the skin injected with lead and holmium chlorides. The X-ray diffraction pattern of the deposits showed three lines (1, 3.41 Å; 2, 3.11 Å; and 3, 2.79 Å) that correspond to the three main lines of apatite. It is probable that the material consists of hydroxyapatite. In areas pretreated with sodium pyrophosphate there was a significant decrease ($P < 0.001$) in calcium and phosphorus when compared with skin treated

TABLE III

Calcium and phosphorus concentrations in blood and urine following the intravenous administration of sodium pyro- and ortho-phosphates

Group	Treatment	Blood*		Urine†	
		Calcium (mg %)	Phosphorus (mg %)	Calcium (μg/90 min)	Phosphorus (μg/90 min)
1	Distilled water	9.60±0.77	6.26±0.16	19±2.4	273±99
2	Sodium pyrophosphate	8.0±0.83	8.19±0.10	25±4.7	1530±112
3	Sodium orthophosphate	10.0±0.17	8.18±0.22	28±6.8	1529±107

*Blood was withdrawn 5 min after treatments.
†Urine was collected 90 min after treatments.
NOTE: The values are averages ± standard errors.

with the chlorides alone; upon X-ray diffraction, no patterns of apatite were seen.

Effect of Sodium Pyrophosphate on the Levels of Calcium and Phosphorus in Normal Rats and Rats Treated with Calcium Acetate, DHT, and Parathyroid Extract

One hundred and sixty rats (200 g body weight) were divided into 11 groups and treated as shown in Tables III–V. In the first series (Table III), we investigated the influence of sodium pyrophosphate and orthophosphate on the calcium and phosphorus levels in both the blood and urine of normal rats. The compounds were given intravenously very slowly under light ether anesthesia, in amounts equivalent to 1.2 mg of inorganic phosphorus, always in 1 ml distilled water. Rats of group 1 received 1 ml of physiologic saline solution for control purposes. Groups 2 and 3 consisted of 20 animals each. From 10 rats we drew blood 5 min after a single injection of sodium pyro- and orthophosphate. The 10 remaining animals were given two injections of the compounds at 30-min intervals; they were placed in metabolic cages immediately after the first injection and urine was collected after 90 min. As seen in Table III, the administration of sodium pyrophosphate caused a significant decrease ($P < 0.001$) in total serum calcium and no change in calcium excretion. Both sodium pyro- and ortho-phosphate increased ($P < 0.001$) the levels of inorganic phosphorus in the serum and urine.

The apparent hypocalcemic effect of sodium pyrophosphate raised the question concerning whether this compound might interfere with the determination of calcium. To resolve this point, an in vitro experiment was performed in which sodium pyrophosphate was added to pooled rat serum in amounts (0.006 to 0.12 mg of P/ml of serum) approximately equivalent to those encountered in the blood of rats treated intravenously with this compound. There was no difference between the calcium content in these samples and control sera. However, after adding sodium pyrophosphate to the serum in amounts higher than 0.06 mg of P/ml. we observed the formation of a precipitate which remained in suspension. After centrifugation (10 min at 3000

PLATE I

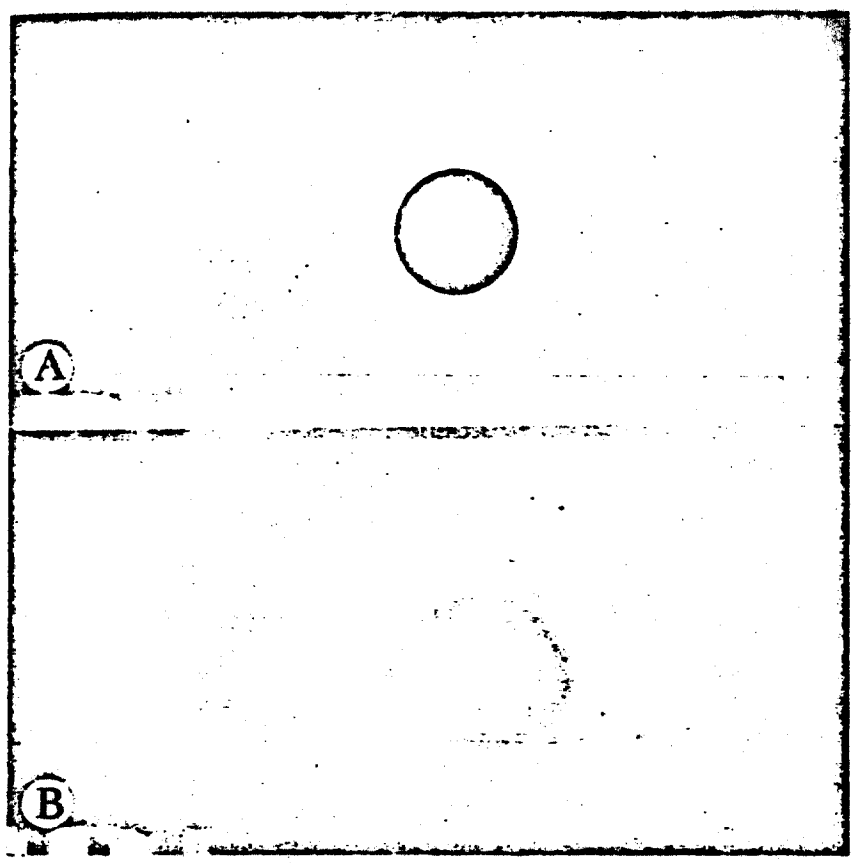

FIG. 1. Action of pyrophosphate on skin calcification induced by lead chloride. (A) X-ray diffraction of a skin treated with lead chloride; pattern characteristic of apatite. (B) X-ray diffraction pattern of a skin treated with lead chloride and sodium pyrophosphate; no apatite pattern.

TABLE IV

Action of sodium pyrophosphate on calcium and phosphorus concentrations after calcium acetate overdosage

Group	Treatment	Blood*		Urine*	
		Calcium (mg %)	Phosphorus (mg %)	Calcium (μg/90 min)	Phosphorus (μg/90 min)
1	None	9.60±0.77	6.26±0.19	14±2.6	328±84
2	Calcium acetate	11.20±0.60	6.70±0.20	137±52	392±126
3	Calcium acetate + pyrophosphate	9.30±0.50	5.30±0.29	28±7.0	366±141

*Blood and urine were collected 90 min after calcium acetate administration.
NOTE: The values are averages ± standard errors.

r.p.m.) of this suspension, the calcium content of the supernatant was significantly decreased when compared with that of control sera. After a single injection of sodium pyrophosphate the hypocalcemic effect was transient. Examinations performed 30 min and 1 h after a single injection showed that serum calcium had returned to normal.

In the second series of experiments, we investigated the effect of sodium pyrophosphate on hypercalcemia induced by calcium overdosage (Table IV). Thirty rats were divided into three equal groups. Animals of group 1 received no treatment. Groups 2 and 3 were given calcium acetate by stomach tube at the dose of 2 mmoles in 2 ml distilled water. After 30 min and 1 h, group 3 received sodium pyrophosphate (in amounts equivalent to 1.2 mg of inorganic phosphorus in 1 ml of distilled water) intravenously very slowly under light ether anesthesia. Urine and blood were collected 90 min after calcium acetate administration to determine calcium and phosphorus contents.

Table IV shows that 90 min after calcium acetate treatment there was a significant increase ($P < 0.005$) in serum calcium in comparison with values found in control rats (group 1). Injections of sodium pyrophosphate significantly decreased ($P < 0.005$) the hypercalcemic effect of calcium acetate. The level of phosphorus was similar in both control and calcium acetate treated rats, but it was lower ($P < 0.001$) in animals receiving both calcium and sodium pyrophosphate as compared with calcium acetate treated rats.

The concentration of calcium in urine was significantly higher ($P < 0.001$) in the group given calcium acetate than in untreated controls. In animals treated with both calcium acetate and sodium pyrophosphate, calcium excretion was normal. Phosphorus excretion was similar in all three groups.

In the third series (Table V), we investigated the action of sodium pyrophosphate upon hypercalcemia caused by DHT and parathyroid extract. Seventy-five rats were divided into five equal groups. Animals of group 1 served as untreated controls. Rats of groups 2 and 3 were given DHT by stomach tube on the first day at the dose of 2.5 mg in 0.5 ml of corn oil. Groups 4 and 5 received parathyroid extract subcutaneously twice on the first and second days at the dose of 150 I.U. Animals of groups 3 and 5 were also injected

TABLE V

Effect of pyrophosphate on calcium and phosphorus concentrations of rats treated with DHT and parathyroid extract

Group	Treatment	Blood*		Urine†	
		Calcium (mg %)	Phosphorus (mg %)	Calcium (mg/24 h)	Phosphorus (mg/24 h)
1	None	10.0±0.14	6.44±0.16	0.480±0.07	12.16±1.22
2	DHT	15.2±0.39	8.11±0.25	6.44±1.44	19.31±1.69
3	DHT + pyrophosphate	12.2±0.22	8.13±0.28	3.78±0.99	22.02±1.92
4	Parathyroid extract	13.3±0.30	6.98±0.44	3.90±0.86	23.58±2.26
5	Parathyroid extract + pyrophosphate	11.3±0.30	8.90±0.61	3.94±0.65	26.60±0.99

*Blood was withdrawn 1 h after the last pyrophosphate injection.
†Urine collection began 24 h after DHT and immediately after the last parathyroid extract administration.
NOTE: The values are averages ± standard errors.

intravenously with sodium pyrophosphate at the dose equivalent to 1.2 mg of inorganic phosphorus at four different time intervals: 22, 23, 46, and 47 h after DHT or the first parathyroid extract treatment. Blood was withdrawn from 10 animals per group 1 h after the last sodium pyrophosphate treatment, and urine collected (for 24 h) beginning 24 h after DHT or immediately after the last parathyroid extract administration. To evaluate morphologic changes, the remaining five animals per group were killed on the sixth day of the experiment.

As shown in Table V, 48 h after the administration of DHT (group 3) and parathyroid extract (group 4), blood calcium was significantly increased ($P < 0.001$). Sodium pyrophosphate decreased ($P < 0.001$) the hypercalcemic effect of both DHT and parathyroid extract. The concentration of phosphorus in the blood was consistently high in rats receiving DHT alone or together with sodium pyrophosphate. In parathyroid extract treated rats, the serum phosphorus was normal, but it increased ($P < 0.001$) in animals treated in addition with sodium pyrophosphate.

Both DHT and parathyroid extract caused ($P < 0.001$) hypercalciuria, and sodium pyrophosphate decreased ($P < 0.001$) the hypercalciuric effect of DHT only. Finally, the administration of DHT and parathyroid extract alone or with sodium pyrophosphate induced comparable hyperphosphaturias.

Histologically, rats treated with DHT or parathyroid hormone and killed on the sixth day of the experiment showed osteitis fibrosa, a typical calcification of the myocardium and aorta (particularly pronounced in DHT-treated animals) as well as calcification of the proximal convoluted tubules of the kidney. Sodium pyrophosphate did not modify these pathologic changes.

Discussion

Our results indicate that sodium pyrophosphate administered subcutaneously inhibits the skin calcification induced by chlorides of iron and chromium in DHT-treated rats and by lead and holmium in normal rats. The chemical determination of calcium and phosphorus as well as the X-ray diffraction

analysis provides further data supporting the view that material deposited at lead and holmium injection sites is hydroxyapatite and not a simple complex formed by the injected metal with an anion such as phosphate or carbonate.

Our observations do not warrant any far-reaching conclusions concerning the mechanism by which sodium pyrophosphate inhibits calcification. It has been assumed that it prevents the growth of apatite crystals (16). Although this mechanism could explain our results, it is also conceivable that sodium pyrophosphate exerts its effect by strongly complexing the various metals.

The present studies also show that in normal rats a single intravenous injection of sodium pyrophosphate induces a transient hypocalcemia without affecting the calcium excretion through the kidney. Similarly, treatment with this compound significantly decreases the hypercalcemias caused by calcium, DHT, and parathyroid extract. Under our conditions, calcium excretion was influenced only in DHT-treated rats. An additional experiment (not reported here) showed that on the sixth day there was no difference between the blood calcium levels of animals receiving DHT or parathyroid extract alone and those which received one of these treatments together with sodium pyrophosphate.

Although other possibilities cannot be excluded, the transient decrease in serum calcium produced by sodium pyrophosphate under our experimental conditions may be explained by the precipitation of a calcium–pyrophosphate complex as proved by our in vitro experiment. Since in pyrophosphate-treated rats the urinary excretion of calcium remains normal, a simple although not yet proven hypothesis may be that the calcium–pyrophosphate complex is absorbed in the reticuloendothelial system. In this connection it is noteworthy that in the rat, the injection of colloidal calcium phosphate is followed by phagocytosis of this material by the macrophages of the liver and spleen and that the retention of the ingested colloidal particles is temporary (17). In our experiment, the administration of sodium orthophosphate (containing the same amount of P as sodium pyrophosphate) failed to produce any change in the total serum calcium. Maybe the calcium–pyrophosphate complex is stronger or more easily phagocytosed than the calcium–orthophosphate complex.

Recently, other investigators (15) have shown that in the rat, concurrent treatment with polyphosphates did not modify the changes in ionic and ultrafiltrable serum calcium induced by vitamin D. Their results appear to contradict our findings (Table IV). However, the decrease in serum calcium observed in our experiment lasted only 1 h following sodium pyrophosphate treatment. The different doses of the compounds administered, as well as the time intervals at which calcium was measured, easily explain the disparity between the results of the two series of experiments. These different conditions may also account for the fact that we did not observe an inhibition by sodium pyrophosphate of the aorta calcification in DHT-treated rats.

We may therefore conclude that sodium pyrophosphate inhibits calcification of the skin produced by various metals in untreated and in DHT-pretreated

rats. It also decreases the blood calcium level (significantly, though only temporarily) in normal animals and in those treated with calcium, DHT, and parathyroid extract.

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переменные: процент снижения засоренности и время, прошедшее после внесения гербицидов.

Для графического выражения этих данных на ординате откладывается снижение засоренности в пробитах, а на абсциссе — логарифмы дней, прошедших после внесения гербицидов. Соединив соответствующие точки, получим графическое изображение гербицидного действия двух форм монурона во времени (рис. 3). На графике показателю NL_{50} соответствует прямая линия, проведенная по точкам «пробит» параллельно абсциссе.

Из табл. 6 видно, что гранулированный препарат по длительности действия превосходит порошковидный. Если при втором учете, данные которого использовались при расчете NL_{50}, гранулированный монурон по эффективности несколько уступал порошковидному, то в динамике действие его возрастало, в результате чего снижение засоренности на 50% наблюдалось даже на второй год после внесения гербицида.

Таким образом, токсичность почвенных препаратов можно характеризовать по совокупности показателей NL_{50} и τL_{50}.

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Химия в животноводстве

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УДК 636.087.7 + 636.5

Новые фосфорные минеральные подкормки для молодняка крупного рогатого скота

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В настоящей статье излагаются результаты опытов по введению в рацион молодняка крупного рогатого скота (порода красно-горбатовская) минеральных фосфорсодержащих подкормок. Опыты проводили в 1964—1965 гг. в колхозе «Панфиловский» Муромского района Владимирской области *.

Кормовой обесфторенный фосфат из Егорьевских фосфоритов ($Ca_3P_2O_8$) — порошок тонкого помола сероватого или светло-коричневого цвета без запаха. По техническим условиям он должен содержать кальция не более 25%, фосфора — не менее 12 и фтора — не более 0,2%.

Кормовой дикальцийфосфат (преципитат, $CaHPO_4$) получают путем смешивания размолотого мела или известняка с термической фосфорной кислотой. Это порошок тонкого помола, белого цвета, по техническим условиям он должен содержать кальция не более 25—27%, фосфора — не менее 19, фтора — не более 0,2, мышьяка — не более 0,012 и свинца — не более 0,005%.

Кормовой динатрийфосфат (двуосновной фосфорнокислый натрий безводный, Na_2HPO_4) — светлая кристаллическая соль, хорошо растворимая в воде, по техническим условиям она должна содержать фосфора не менее 21%, натрия — не менее 13,1, фтора —

* Препараты получены из НИУИФ.

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не более 0,2%. Препарат пригоден для подкормки только жвачных животных.

Кормовой диаммонийфосфат $[(NH_4)_2HPO_4]$ получают путем нейтрализации фосфорной кислоты газообразным аммиаком. Это белый с желтизной кристаллический порошок с запахом аммиака, хорошо растворяется в воде. По техническим условиям он должен содержать фосфора не менее 23%, азота — не менее 20, фтора — не более 0,2 и мышьяка — не более 0,012%. Применяется только в кормлении жвачных животных как фосфорно-азотистая подкормка.

В 1964 г. опыт проводили на трех группах животных (по 40 голов в каждой). Продолжительность опыта — 7 месяцев. Все животные получали одинаковый основной рацион (сено луговое окское, трава луговая сеяных трав, силос кукурузный, корнеклубнеплоды, концентраты) и следующие минеральные подкормки:

Группа животных	
I	кормовой диаммонийфосфат
II	кормовой динатрийфосфат
III	кормовой обесфторенный фосфат из Егорьевских фосфоритов

При постановке на опыт средний живой вес одной головы был 113 *кг*, за 216 дней опыта привес в I группе составил 175,7 *кг*, во II — 173,4 и в III — 165,6 *кг* на 1 голову. Затраты кормов на 1 *кг* привеса были минимальными в I группе (табл. 1).

ТАБЛИЦА 1

Затраты кормов на 1 *кг* привеса

Группа животных	Корм. ед. *кг*	Переваримый протеин, *г*	Кальций, *г*	Фосфор, *г*	Концентраты *кг*
I	7,65	902	91	48	2,38
II	7,87	920	94	50	2,38
III	8,32	974	103	54	2,50

Балансовый опыт провели в зимний период. Рацион был следующим (*кг* на 1 голову в сутки):

Сено луговое окское	2,5
Дерть ячменная	3,0
Картофель вареный	5,0
Свекла сахарная	5,0
Фосфорная минеральная подкормка	0,050

Переваримость питательных веществ рациона была хорошей. Сырой протеин, жир и клетчатка несколько лучше переваривались в организме животных, получавших динатрийфосфат (табл. 2).

ТАБЛИЦА 2

Коэффициент переваримости питательных веществ

Группа животных	Сухое вещество	Органическое вещество	Сырой протеин	Сырой жир	Сырая клетчатка	ВЭВ
I	62,9	67,0	59,3	36,1	35,8	76,2
II	66,3	69,5	62,9	49,1	39,1	76,3
III	65,3	69,1	55,3	34,1	40,5	78,0

Баланс азота, кальция и фосфора у животных всех групп был положительным (табл. 3).

ТАБЛИЦА 3

Баланс азота, кальция и фосфора

Группа животных	Принято с кормом *г*	Выделено, *г*			Баланс
		с мочой	с калом	всего	
		Азот			
I	122,8	31,4	56,36	87,7	+35,0
II	108,1	25,1	41,5	66,5	+41,6
III	102,2	24,36	45,86	70,2	+31,6
		Кальций			
I	17,83	0,19	17,02	17,21	+0,12
II	17,21	0,20	11,85	12,05	+5,16
III	23,92	0,21	21,85	22,06	+0,35
		Фосфор			
I	31,96	4,2	24,5	28,7	+3,36
II	24,31	4,3	16,2	20,5	+3,81
III	23,02	2,7	16,7	19,4	+3,62

После окончания опыта животных сдали на мясокомбинат. Все туши и внутренние органы были тщательно осмотрены ветврачами мясокомбината. Никаких отклонений от нормы обнаружено не было. Для контрольного забоя из каждой группы взяли по три животных. Забивали телят после суточной голодной выдержки. Упитанность животных была выше средней. Все туши отнесли к первой категории (табл. 4).

ТАБЛИЦА 4

Показатели убойного выхода

Группа животных	Живой вес после суточной голодной выдержки, *кг*	Вес туши (мясо на костях+сало+мясная обрезь) *кг*	Убойный выход, %
I	256,3	141,6	55,24
II	234,0	130,04	55,55
III	237,3	128,5	54,11

После суточного охлаждения туши были подвергнуты обвалке и жиловке, мясо разде-

лали по сортам. От телят, которым скармливали динатрийфосфат (II группа) и диаммонийфосфат (I группа), мяса высшего, жирного и первого сортов получили 50,0 и 48,6% соответственно, от телят III группы, получавших обесфторенный фосфат, — 45,1%.

В 1965 г. провели второй научно-хозяйственный опыт (продолжительность — 6 месяцев) на трех группах молодняка по 40—45 голов в каждой. Первая группа в дополнение к основному рациону получала кормовой дикальцийфосфат, II — кормовой диаммонийфосфат и III — кормовой динатрийфосфат. При постановке на опыт средний живой вес 1 головы составлял 125 *кг*, в конце опыта показатели были следующие:

Группа животных	Живой вес 1 головы *кг*	Привес за период опыта *кг*
I	271,5	146,5
II	265	140,0
III	261	136,0

Случаев заболевания молодняка от постоянного скармливания фосфорных подкормок за время этого опыта (как и первого) не было. Животные хорошо росли, привесы были высокие. Самые низкие затраты на 1 *кг* привеса были у молодняка I группы, получавшей дикальцийфосфат (табл. 5).

ТАБЛИЦА 5

Затраты на 1 *кг* привеса

Группы животных	Корм. ед.	Переваримый протеин, *г*	Кальций *г*	Фосфор *г*
I	6,98	684	68	50
II	7,31	786	62	29
III	7,52	809	64	50

В 1965 г. летом провели балансовый опыт. В период опыта рацион был следующим (*кг* на 1 голову в сутки):

Овес зеленый	20
Картофель сырой	5
Комбикорм	2,5
Фосфорная минеральная подкормка	0,060

Переваримость питательных веществ летнего рациона телятами всех групп была выше, чем зимнего (табл. 6).

У всех животных был довольно высокий положительный баланс азота, кальция и фосфора. Из летнего рациона лучше, чем из зимнего, усваивались в организме животных азотистые и минеральные вещества.

ТАБЛИЦА 6

Коэффициенты переваримости питательных веществ

Группа животных	Сухое вещество	Органическое вещество	Сырой протеин	Сырой жир	Сырая клетчатка	БЭВ
I	78,5	80,6	73,3	54,6	81,3	83,7
II	78,4	80,4	72,8	60,4	80,7	83,5
III	76,1	78,9	72,3	62,4	78,8	82,4

В конце опыта все поголовье молодняка было забито на Муромском мясокомбинате. Никаких отклонений от нормы в развитии внутренних органов не обнаружено. Для контрольного забоя из каждой группы взяли по три головы. Упитанность всех животных была выше средней, убойный выход был очень высоким (табл. 7).

ТАБЛИЦА 7

Показатели убойного выхода

Группа животных	Живой вес после суточной голодной выдержки *кг*	Вес туши (мясо на костях+сало+мясная обрезь), *кг*	Убойный выход %
I	277	155,6	56,1
II	266	159,0	59,7
III	236	140,9	59,2

После суточного охлаждения туши были подвергнуты обвалке и жиловке, мясо разделили по сортам. От животных II и III групп получили мяса высшего плюс первого сорта 38,7 и 41,9% соответственно, от животных I группы — 36,5%.

Выводы

1. Новые фосфорсодержащие минеральные подкормки — кормовые дикальцийфосфат (преципитат), диаммонийфосфат, динатрийфосфат, обесфторенный фосфат из Егорьевских фосфоритов — животные поедали охотно; ни одного случая заболевания, связанного со скармливанием фосфатов, не было.

2. Балансирование рационов молодняка по фосфору и кальцию путем введения кормовых фосфатов способствовало получению высоких привесов.

3. Животные лучше всего использовали кальций из рационов, содержащих дикальцийфосфат, а фосфор — из рационов с диаммонийфосфатом и динатрийфосфатом.

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The Metabolic Behaviour of Phosphorus.

Balance Sheets of Phosphorus in Full-grown Rats Fed with Tri-stearine and Ammonium Phosphate.

By A. WESTERLUND.

From the Physiological Institute.

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Much work has already been done on the metabolic behaviour of phosphorus in the animal body. Numerous balance sheets of this element in different species and in different chemical compounds are given in the literature. They cannot be reviewed here. Nevertheless, continued research is necessary to formulate general rules for the metabolism of the element in question. Especially such investigations as deal with phosphorus from different sources, e. g. from various food-stuffs, are not so frequent. Furthermore, the bulk of recent work on phosphorus metabolism is mainly directed towards the conditions in young growing animals, for instance the extensive literature dealing with rhachitidogenic diets.

The present investigation takes up the problem from a somewhat special point of view. It deals only with adult animals living under quite special conditions. The leading feature of these is that the animals should be in a state of calcium starvation, i. e. they should receive such restricted amounts of calcium with the food that the loss of this element with the intestinal excreta should exceed the intake. This negative calcium balance should be still more accentuated by giving the animals a kind of fat which augments the fecal

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loss of calcium. For this purpose pure tri-stearine was chosen. Finally, all such components should be absent from the experimental diets as are suspected to decrease the demand of calcium. Therefore, the diets should lack vitamin D (cf. Nicolaysen 1937).

The source of phosphorus used in the present investigation was ammonium phosphate ($(H_4N)_2HPO_4$) delivered from Kahlbaum in Berlin. The author aims at extending the investigation by using other sources of phosphorus also, in particular some common food-stuffs. Among them, milk attracts special attention in comparison with bread, for instance. Certain of these additional investigations are on record.

In addition to a presentation of complete balance sheets for phosphorus at different levels of intake, and of calcium as well, the present investigation intends to gain information as to the factors which control the elimination of phosphorus (and calcium) with the solid and liquid excreta.

The experimental animals were seven male albino rats of varying body weight but surely practically full-grown. They are referred to in the following by Arabic numerals. Among them, five, i. e. Nos. 3—7, belonged to our own colony. They had received our stock food since the weaning. The remaining two were purchased, but had been kept in our *vivarium* for some weeks prior to the experiment so that they may be considered well accustomed to the stock food. The body weights of the animals are given in Tab. 1.

Tab. 1.

The body weights of the animals (kg).

1	2	3	4	5	6	7
.185	.216	.301	.225	.241	.212	.345
.180	.216	.297	.228	.241	.212	.330
.180	.211	.289	.225	.242	.210	.320
.169	.213	.293	.220	.236	.208	.322
.170	.217	.290	.221	.237	.209	.312
.177	.215	.295	.224	.239	.210	.326

The bottom line gives the means.

The experimental period covered 10 days, the first 5 of which formed a preparatory part intended to accustom the rats to the experimental diets, and during which the food intake was recorded and adjusted. The actual test period, during which the excreta were collected as well, began with the 6th day and lasted 5 days. During the whole experimental period the animals were kept in metabolic cages of our own pattern (1929), the frame consisting of aluminium, the sides of wire net, and the floor of parallel glass tubes, resting on a pyramid-shaped funnel of tinned copper. The separation of solid and liquid excreta was effectuated by a cone of silver grating through which the urine could flow and from which the fecal boli rebounded. The daily weighings were carried out with the animal enclosed in the cage.

Before the experimental period, the rats were kept in a basement room, in a large common cage with plenty of wood-wool which was changed frequently. The stock food was the same as in a previous experiment (1934), mainly consisting of crushed wheat, skim milk cheese, and carrots. The components were separate and accessible *ad libitum*. Its content of vitamin D was probably very small.

The experimental diets. The basal food had the following composition:

hemp-seed edestin	14 parts,
rice starch	58 »
paper pulp	17 »
tri-stearine	9 »
salt mixture	2 »

In comparison with basal foods previously used in this laboratory, the present one contained about 3 times more paper pulp. This increase had been made in order to guarantee the fecal boli a suitable consistency, especially in the case of the large phosphorus ingestions. The paper pulp was washed with hydrochloric acid and distilled water in order to reduce the

content of calcium. The salt mixture was the same as in previous experiments, but the calcium phosphate had been excluded.

Regarding the preparation of the experimental food-mixtures the following is to be noticed. The components enumerated above, except the fat, were pulverized and weighed out in an air-dry condition. About a fourth of the starch was gelatinized with hot water in order to give the ready-made food-boli a sufficient coherency. The remaining components were mixed, soaked with tepid water to a dough, and formed in small pieces. After drying, these were ground to a fine powder, into which the following components were incorporated: the gelatinized starch, the fat, different quantities of ammonium phosphate, a constant amount of suction-filtered brewery yeast previously stirred up in a small quantity of hot water, and finally, 1,00 g of citric acid. The mixture was soaked up with tepid water to a paste of suitable consistency and kneaded thoroughly. The resulting mass, moist but not sticky, was weighed, formed to boli of convenient size and stored hermetically in a refrigerator. A weighed quantity of these food-boli were offered to the animals thrice daily, placed directly upon the bottom of the cage. The risk that minute pieces from them should be dropped and slip through the grating of the urinary separator was next to none. The moisture of the food-boli was determined daily.

It follows from this method of preparation that losses of food-ingredients could not be avoided. The existence of such losses becomes obvious from differences in the total weight of the individual food-mixtures actually recorded. Therefore, variations in the composition *between* the food-mixtures are to be expected. Furthermore, the homogeneity *within* the ready-made food-mass may leave something to be desired.

In addition to the presence of tri-stearine, the experimental diets are characterized by their complete lack of vitamins A and D.

The methods of chemical analyses were essentially the same as in previous investigations from this laboratory. Objects of direct analysis were in the present investigation the following elements: Ca, Mg, K, Na, P, S, Cl. Only the contents of Ca and P are referred to in the following discussion. The percentages of the other elements analysed show a highly significant covariation with the percentages of Ca. The percentages of fat were obtained by calculation, on the basis of the dry weight of the food-mixtures and the weighed quantity of the fat. Consequently, the accuracy of the data for the consumption of tri-stearine is less than in the case of direct determination.

Controls of the Chemical Analyses.

As the present investigation intends to determine several quantitative relations, it was considered useful to test the results of the chemical analyses by a series of controls. The different experimental diets are referred to in the following by Roman numerals, in such a way that the diet of rat 1 is called »food-mixture I», the diet of rat 2 »food-mixture II» etc. In most cases, the components of the different food-mixtures came from a large supply procured at the beginning of the experiments. The starch and the yeast represent exceptions, the former originating from a special batch in food-mixtures I and II, the latter being delivered fresh for every separate mixture.

Tab. 2.

The percentages of Ca.

Food-mixtures	I	II	III	IV	V	VI	VII
Phosphate added, g	1.000	2.000	.501	.000	1.502	1.001	4.000
Dry weight, g	319	320	326	327	322	323	298
Ca-percentages	.0162 .0161 .0167	.0168 .0164 .0166	.0215 .0215	.0194 .0199	.0208 .0200	.0216 .0211	.0239 .0231
Means	.01633	.01660	.02150	.01965	.02040	.02135	.02350

We begin with the controls of Ca. The analyses obtained for this element, reduced to a dry basis, are given in Tab. 2, which shows the phosphate additions and the dry weights also. Tab. 2 indicates that the triple analyses for food-mixtures I and II have come out with lower values than the double analyses for the remaining mixtures. The difference between the means is highly significant, for — on a basis of per ten thousand and using R. A. Fisher's (1930) notations — the following relations exist:

$$s^2(1/6 + 1/10) = .003449,$$

$$n = 14$$

$$t = 26; \; t_{min.} = 3.$$

On the other hand, an analysis of variance relating to the Ca values in food-mixtures III—VII indicates that the variation in Ca percentages between the mixtures is considerably wider than within them, as is shown in Tab. 3.

Tab. 3.

Analysis of variance of the Ca percentages in food-mixtures Nos. III—VII.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	S. D.	log. nat. 100 S. D.
within the mixtures	5	.00890	.001780	.0422	1.14
between the mixtures	4	.16826	.042065	.6497	4.17
total	9	.17716			$z = 2.73$
					$z_{min.} = .82$

In fact, the difference is highly significant. This considerable discrepancy between the inter-class and intra-class variations was hardly expected. A possible explanation is that the salt mixture and phosphate preparation used contained accidental impurities of Ca. If this is the case, we may expect the Ca analyses to show a negative covariation with the corresponding dry weights of the food-mixtures, and

a positive covariation with the addition of phosphate. A direct calculation of the regression coefficients strengthens the assumption. If the Ca analyses are adjusted by regression to a weight of 319 g and to an addition of phosphate of 1.101 g by using the regression coefficients obtained, we get the figures in Tab. 4.

Tab. 4.

Ca percentages in food-mixtures Nos. III—VII adjusted by regression to a dry weight of 319.2 g and a phosphate addition of 1.1008 g.

III	IV	V	VI	VII
.0222	.0203	.0210	.0220	.0217
.0222	.0208	.0202	.0215	.0209

If this new set of computed Ca analyses is put through an analysis of variance, the discrepancy between the inter-class and intra-class variations comes out much smaller than before, the difference (z) taking the value of .88. But this diminution cannot be considered sufficient, as the corresponding probability is still too high, the value of z not being permitted to exceed .82. The remaining inconsistency, therefore, may depend on unrecorded dissimilarities in the preparation of the food-mixtures.

Tab. 5.

P percentages in food-mixtures Nos. I and II.

Food mixtures	I	II
Phosphate added, g	1.00	2.00
Dry weight, g	319	320
P-percentages	.1161 .1111 .1111	.1882 .1891 .1908
Means	.11297	.18937

The next control relates to P. First, the analyses for food-mixtures I and II are given (Tab. 5). If the P percentages in a phosphate-free mixture of the same composition is extrapolated, we get .0366 per cent. The P contents coming from

the addition of phosphate are thus (.1130 — .0366 =) .076 and (.1894 — .0366 =) .153 per cent. respectively. Supposing the P content in the phosphate preparation to be 23.45 g in 100 g, the absolute quantities of P which come from the additions of ammonium phosphate amount to .2345 och .4690 g, resp., and their P percentages

in food-mixture I 074,
in food-mixture II . . . . . .15 .

These values agree fairly well with those obtained by direct chemical analysis and extrapolation.

The P percentages of the remaining 5 food-mixtures are found in Tab. 6. The bottom line gives the values which

Tab. 6.

P percentages in food-mixtures Nos. III—VII.

Food mixtures	III	IV	V	VI	VII
Phosphate added, g	.501	.000	1.502	1.001	4.000
Dry weight, g	326	327	322	323	298
P-percentages	.0614 .0616 .0598	.0252 .0262 .0251	.1328 .1324 .1322	.0897 .0935 .0941	.2986 .3080 .3243
Means	.06093	.02550	.13250	.09243	.31030
Means adjusted by regression	.05935	.02348	.13125	.09538	.31220

have been adjusted by regression, using the weights and the phosphate additions as argument variates. The next table (Tab. 7) gives the P percentages in the same food-mixtures.

Tab. 7.

P percentages adjusted by regression to a dry weight of 319.2 g and a phosphate addition of 1.4008.

III	IV	V	VI	VII
.1264	.1261	.1259	.1186	.1107
.1266	.1271	.1255	.1224	.1201
.1248	.1260	.1254	.1220	.1364
.12593	.12640	.12560	.12133	.12240

but now adjusted to a common weight and to the same addition of phosphate. The pertaining analysis of variance suggest that the variation between the food-mixtures cannot be considered significantly wider than within them, but this result seems to depend upon the considerable intra-class variation in food-mixture VII only. For, if its values are excluded from the analysis of variance, the inter-class variation comes out significantly wider than the intra-class variation. Therefore, just as in the case of Ca, there must have been unrecorded differences in the preparation of the 5 food-mixtures in question.

The second control consists in a comparison between the P percentages calculated as above and those obtained by direct chemical analysis. The first link, i. e. the calculation, is given in Tab. 8. The second link is shown in Tab. 9. The values

Tab. 8.

P percentages computed directly from the dry weights of the food-mixtures and the phosphate additions.

Food mixture	Phosphorus added g	Phosphate Phosphorus per cent.
IV	.0000	—
III	.1173	.036
VI	.2345	.073
V	.3518	.109
VII	.9380	.315

Tab. 9.

P percentages adjusted by regression and obtained by chemical analysis.

Adjusted by regression		Obtained by analysis	
Total P	Phosphate P	Total P	Phosphate P
.0235	—	.0255	—
.0594	.036	.0609	.035
.0954	.072	.0924	.067
.1313	.108	.1325	.107
.3122	.289	.3103	.285

in the second and fourth columns were obtained by subtracting the value for mixture IV, to which no phosphate had been added, from the other values of total P.

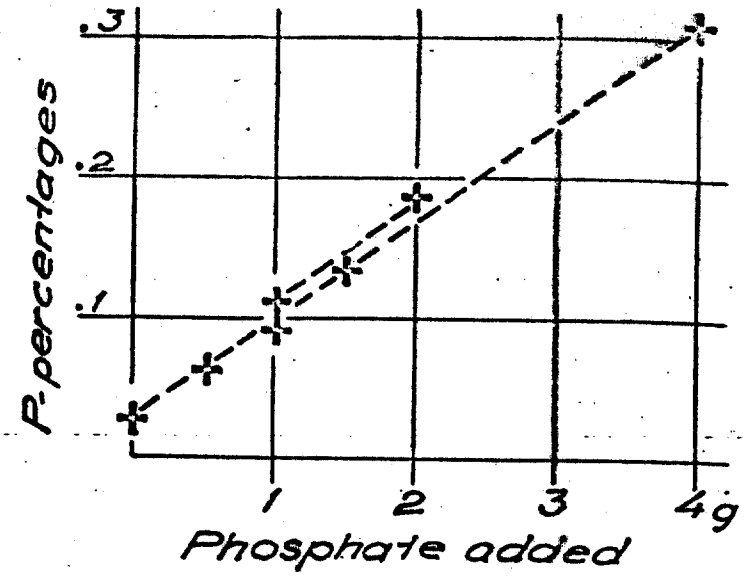

Fig. 1. The gross regression of P percentage upon the phosphate addition.

The accuracy of the whole set of P analyses is illustrated in Fig. 1.

The regression lines seem to represent the observations very well. The shorter line refers to food-mixtures I and II. Their proportionality factor does not differ significantly from the regression coefficient of the rest.

The Elimination of Calcium.

The intestinal elimination. The values for fecal calcium are shown in Tab. 10. The top line gives the numbering of the

Tab. 10.

Fecal calcium elimination in the individual rats (cg.

1	2	3	4	5	6	7
.95	.74	1.03	1.19	1.33	1.29	1.90
.96	1.24	1.49	1.36	1.46	1.60	1.85
1.08	1.15	1.18	1.80	1.45	1.77	1.62
1.20	1.21	1.49	1.74	1.87	1.64	1.72
.98	1.66	1.87	1.23	1.59	1.72	2.27
1.034	1.206	1.412	1.461	1.540	1.604	1.872

animals, the bottom line the means. The pertaining analysis of variance suggests that the variation between the animals is significantly wider than the variation within them (Tab. 11).

Tab. 11.

Analysis of variance of fecal calcium elimination.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	S. D.	log. nat. 10 S. D.
within the animals	28	1.7824	.0636	.2523	.925
between the animals	6	2.2204	.3701	.6084	1.806
total	34	4.0028			$z = .88$ $z_{min.} = .63$

One of the problems of the present investigation is to find the factors which cause this considerable inter-class variation.

Alimentary factors and the weights of the rats. At first, some purely alimentary factors were tested. Table 12 contains the

Tab. 12.

Food ingested, dry substance in dekagram.

1	2	3	4	5	6	7
1.12	.94	.95	1.14	1.09	1.01	1.34
1.01	1.06	1.17	1.09	.96	1.14	1.35
1.04	1.17	1.28	1.03	1.20	1.18	.92
1.05	1.34	1.53	.85	.93	1.06	1.03
1.25	1.52	1.61	.88	1.27	1.11	1.13
1.094	1.206	1.308	.998	1.090	1.100	1.154

figures for total food consumption. The analysis of variance gives the difference (z) a value of .24, while the 5 per cent probability requires .63. Therefore, the inter-class variation cannot be considered significantly wider then the intra-class variation.

The next step is to determine whether the dissimilarities in *percentages* of calcium and of fat between the different food-mixtures modify the result of the analysis of variance, when the *absolute amounts* of intake of the components mentioned

are considered. The values of these consumptions are given in Tab. 13 and Tab. 14.

Tab. 13.

Calcium consumption (cg).

1	2	3	4	5	6	7
.18	.16	.20	.22	.22	.22	.31
.16	.18	.25	.24	.20	.24	.32
.17	.19	.28	.20	.21	.25	.22
.17	.22	.33	.17	.19	.23	.24
.20	.25	.35	.17	.26	.24	.27
.176	.200	.282	.194	.222	.236	.272

Tab. 14.

Fat consumption (g).

1	2	3	4	5	6	7
1.05	.88	.87	1.05	1.02	.94	1.35
.95	.99	1.08	1.00	.89	1.06	1.36
.98	1.10	1.16	.94	1.12	1.10	.93
.99	1.26	1.41	.78	.87	.98	1.01
1.18	1.43	1.48	.81	1.18	1.03	1.14
1.030	1.132	1.200	.916	1.016	1.022	1.161

The new analyses of variance show that this is the case for the consumption of calcium only, the difference (z) taking the following values:

for the consumption of calcium 94,
" " " fat 31,
minimum value . . . . . .63.

The alimentary factor which above all may be assumed to exert an obvious influence upon the intestinal elimination of calcium is the phosphorus in the food. The experimental data for the consumption of this element are given in Tab. 15. It follows from the experimental program that the inter-class variation in this case is very much wider than the intra-class variation.

Thus, among the factors so far mentioned, there are only three which show a wider inter-class than intra-class variation.

Tab. 15.

Phosphorus consumption (mg.)

1	2	3	4	5	6	7
1.27	1.78	.58	.29	1.14	.93	4.45
1.14	2.02	.71	.28	1.27	1.02	4.19
1.17	2.21	.78	.26	1.38	1.09	2.85
1.19	2.54	.92	.22	1.23	.98	3.19
1.41	2.88	.98	.22	1.68	1.03	3.51
1.236	2.286	.794	.254	1.110	1.016	3.578

viz. the consumption of calcium and phosphorus and, as seen from Tab. 1, the body weights of the rats also. Our problem, therefore, represents a multiple covariation, and it is solved in the usual way by calculating net regression coefficients according to the principle of least squares, after a successive introduction of the three variates to be examined into the system of observation equations. The result of this calculation is seen in Tab. 16.

Tab. 16.

Net regression coefficients for fecal calcium elimination as dependent variate.

Body weight	3.8 ± 1.6 2.4 4.0	2.8 ± 3.9 .7 4.6	2.2 ± 5.1 .4 5.8
Ca ingested.		1.5 ± 5.0 .3	1.9 ± 6.1 .3
P ingested			.02 ± .12 .2

Tab. 16 is divided in squares. The upper line of each square gives the net regression coefficients and their mean error; the lower line presents the values of the quotient (t): the first figure is the value observed, the second the value required for odds 99 against 1 that the coefficient may be regarded as greater than zero. It is seen in Tab. 16 that none of the argument variates included, not even the consumption of phosphorus, comes up to this requisite.

It remains to examine whether the experimental factor which did not show a sufficiently wide inter-class variation, viz. the consumption of fat, may be connected with the variation of

fecal calcium *within* the animals. The regression coefficient takes a positive sign: it is true, but the value is only about 2.2 times its mean error. The corresponding probability is in the region of .05 and may not be considered sufficiently significant, though the tendency to covariation is unmistakable. The poor significance may depend on the narrow variation range of the fat ingestion and on inexactitudes of the fat values. The question will be discussed in a subsequent paper of this series.

Other factors. The next step is to determine the relation between the fecal calcium elimination and some other, more endogenous factors. One of these is the renal elimination of calcium. It should be noticed that the incompleteness of the collection and the small amounts of urine render the values of urinary calcium somewhat uncertain. Only the inter-class variation can be given (Tab. 17).

Tab. 17.

Animal	Urinary Ca cg	Fecal Ca cg
1	.023	1.034
2	.042	1.206
3	.034	1.412
4	.039	1.464
5	.031	1.540
6	.037	1.604
7	.050	1.872
Mean	.037	1.447

The value of the quotient (t) for the regression coefficent does not exceed 2 and, consequently, may not be considered significantly greater than zero. Therefore, a relation between the urinary and the fecal calcium cannot be demonstrated.

Tab. 18.

Fecal phosphorus elimination (cg).

1	2	3	4	5	6	7
.22	.15	.40	.28	.31	.31	.66
.21	.62	.46	.57	.33	.42	1.20
.17	.26	.39	.43	.28	.51	1.01
.39	.32	.45	.53	.39	.49	.69
.31	.40	.62	.27	.31	.57	1.22
.260	.350	.464	.416	.324	.460	.956

The second factor is the fecal phosphorus. The experimental data for the elimination of this element with the intestinal excreta are shown in Tab. 18.

The pertaining analysis of variance gives the difference (z) a value of 1.2, from which follows that the variation of the

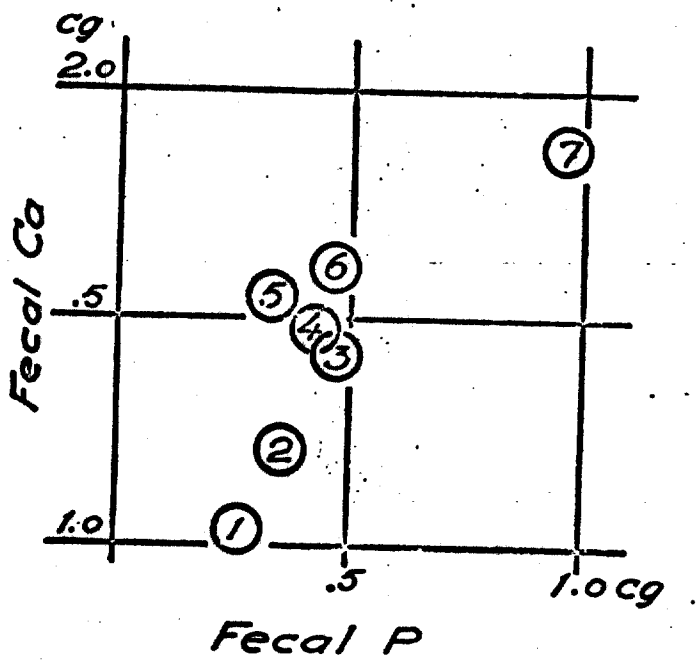

Fig. 2. The corresponding values of fecal phosphorus and fecal calcium.

means is significantly wider than the variation from day to day. The correspondence of the two variates in question is illustrated in Fig. 2.

No significant covariation can be proved, because the value of t does not exceed 3 times its mean error, the corresponding probability being in the region of .03 only. Nevertheless, a tendency to covariation should not be overlooked. A glance at the Figure may suggest a curved regression line, but the introduction of the squared values of the argument variate does not improve the significance.

One of the numerical analyses in the preceding sub-paragraph indicates that the intra-class variation of the fat consumption cannot be considered responsible for the variations in the fecal elimination of calcium *within* the cats, because the significance of the regression coefficient is too poor. It should be tested whether this significance can be enriched by an additional introduction of the fecal phosphorus into the system of observation equations referring to the condition *within* the animals. This is not the case. Neither the fat consumption nor the fecal phosphorus elimination comes out with a significant regression coefficient, when both of these argument variates are included.

The result of the numerical analysis hitherto performed indicates that not a single one of the experimental factors examined can be made responsible for the considerable variation in the intestinal elimination of calcium. An interesting fact is that the wide variation in the consumption of phosphorus is not followed by a variation in this elimination. A possible reason for this is the presence in the food and the intestinal contents of a component like tri-stearine which may eliminate the influence of other factors, owing to its capacity of forming insoluble soaps with calcium.

The renal elimination. It has already been implied that the collecting of urine is more difficult than the collecting of feces. It may be well to consider this condition a little more. The grating of the urinary separator may be liable to cause some sprinkling, but this was practically avoided by placing the urine receptacle in a high beaker. A more serious objection may be that the urine adheres to the glass tubes at the bottom of the cage and dries up there. The urine was collected after every 24 hour period and the inside of the funnel-shaped support together with the separator and the inside of the beaker was washed and the wash water and the urine united for analysis. But the washing of the glass tubes was performed after the 5th and 10th day only. Consequently

the urinary constituents, which refer to the last experimental day proper, may be obtained in greater quantity than those referring to the other days. Therefore a partition of the urinary calcium into interclass and intraclass categories was not possible.

The daily output of urinary calcium has already been tabulated (Tab. 17). The values were obtained by adding the calcium quantities from each day and divide the sum by the number of days (5). Consequently, they represent arithmetical means, the numerical structure of which is not known. Tab. 17 shows that the figures are low, in no case exceeding .5 mg. and rather uniform. If they are placed as dependent variates in a system of observation equations in the usual way, using all available concomitant data as independent variates, the result is that none of these factors shows a significant covariation with the urinary calcium. This result may be regarded as somewhat surprising. Assuming that great quantities of ammonium phosphate in the food should be liable to cause a decrease of the alkali reserve of the blood and, consequently, an increase of the urinary calcium we should rather have expected a demonstrable relation between the phosphorus consumption and the urinary output of calcium.

Attention should be called to another point in this connection, namely the participation of the kidneys in the total elimination of calcium. It is derived from Tab. 17 that the mean of this participation accounts for 2.47 per cent only, with the moderate variance of .206.

Phosphorus.

The intestinal elimination. Turning now to the main object of the present investigation we refer in the first place to the contents of phosphorus in the solid excreta, tabulated above (Tab. 18). The analysis of variance indicates that the variation between the animals is considerably wider than within them. But this result is due to the profuse elimination in rat 7 only,

for if the values from this animal are excluded from the analysis of variance, the significance of the difference (z) is lost.

Just as was done with regard to the elimination of calcium above, our object in this paragraph is to study the conditions which govern the fecal elimination of phosphorus. The study

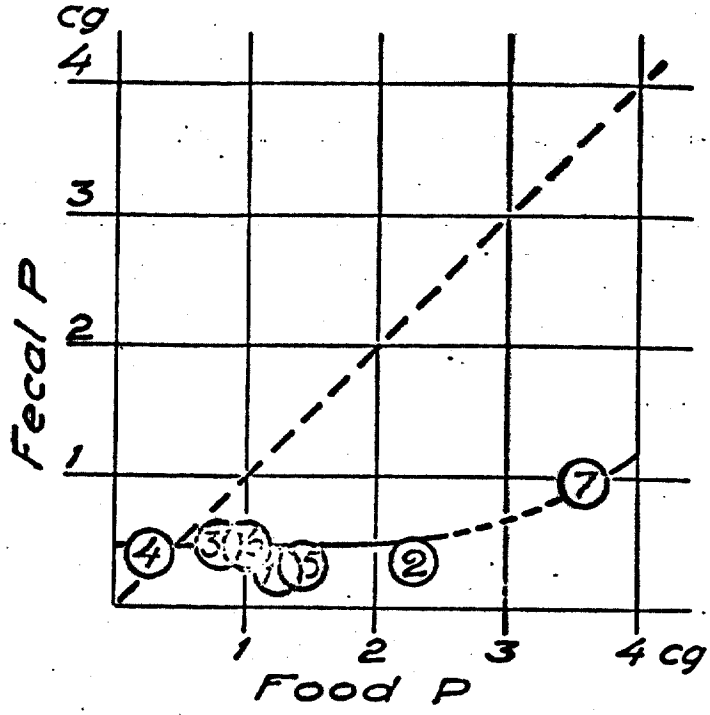

Fig. 3. The corresponding values of food phosphorus and fecal phosphorus. The regression line has been drawn by eye.

has led to the conclusion that none of the concomitant factors recorded shows any significant covariation with this elimination, possibly with the exception of fecal calcium, which has already been mentioned. The result may be regarded as remarkable. This is especially true of the ingestion of phosphorus, as this variate was expected to present a demonstrable relation with the fecal elimination of phosphorus. The alleged correspondence is illustrated in Fig. 3.

A glance at the Figure suggests a curved regression line. A direct attempt to fit a second degree parabola has failed it is true, but this failure seems to depend essentially upon the narrow variation range of the phosphorus intake. Fig. 3 points to the conclusion that — up to an intake of about 20 mg — no connection seems to exist between the oral consumption and the intestinal elimination of the element in question, but that a positive covariation may begin above this limit. If such is the case, the relation may exemplify the rule of the oblique-angled hyperbola proposed by the present author for the regression of fecal output of *calcium* upon the corresponding intake of this element (1938). This rule implies a limit of resorption for phosphorus, as for calcium, which means that, if the corresponding intake exceeds a certain value, a constant quantity is absorbed, irrespective of the size of the intake. The present observation series does not permit of a location of this limit. Probably, it is considerably higher than 40 mg.

Fig. 3 shows a detail of additional interest, relating to rat 4, which had received pure basal food, i. e. without addition of ammonium phosphate. The intake of this animal was 2.5 mg only. The corresponding quantity of fecal phosphorus amounted to 4.2 mg. This shows that the elimination of phosphorus with the intestinal excreta can exceed the simultaneous intake of this element, provided that the intake is sufficiently low.

The renal elimination. It remains to seek the concomitant factors which control the urinary elimination of phosphorus. The experimental data, in the form of means for the different rats, are given in Tab. 19.

The usual numerical analysis indicates that one single experimental factor shows a significant covariation with the renal elimination of phosphorus, viz. the consumption of phosphorus. The corresponding regression coefficient amounts to about 17 times its mean error. The covariation is illustrated in Fig. 4.

Tab. 19.

Urinary phosphorus elimination (cg).

Animal	Urinary P
1	2.030
2	2.772
3	1.296
4	.580
5	2.170
6	1.374
7	4.738
Mean	2.137

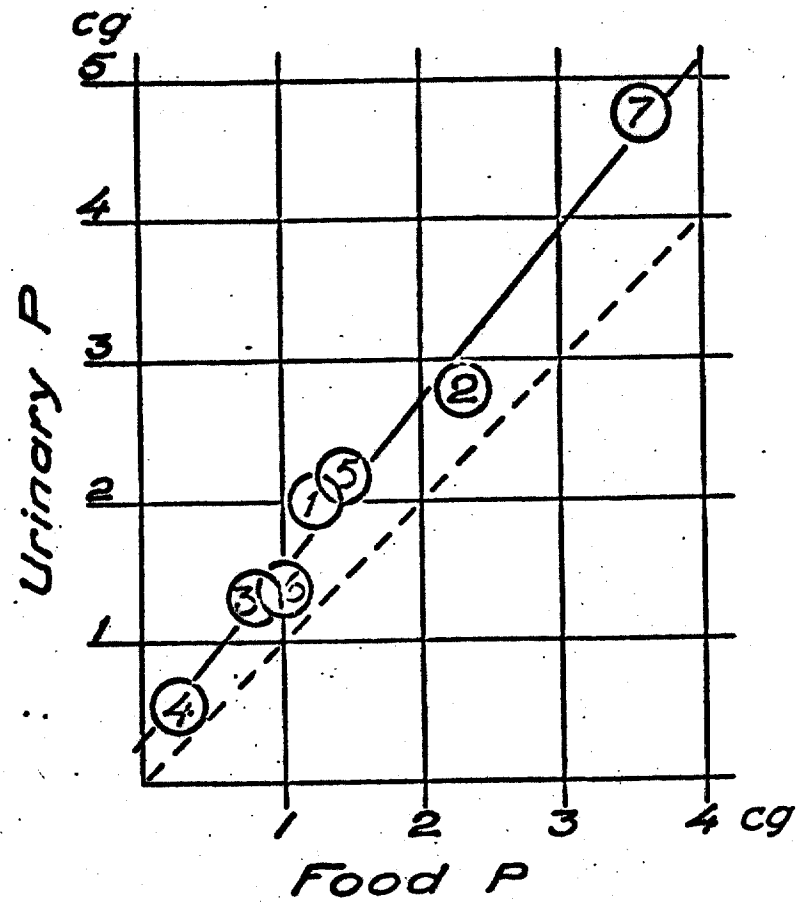

Fig. 4. The regression of urinary phosphorus upon food phosphorus.

The absolute value of the regression coefficient is 1.2. Consequently, the quantities of phosphorus which are secreted by the kidneys increase more rapidly than the corresponding increase of the phosphorus consumption. This is shown graphically in Fig. 4 by the difference in slope between the diagonal, with a regression coefficient of 1.00, and the regression line in question. But the difference between the regression coefficients of these two lines (.2) is only 2.8 times its mean error. Therefore, the corresponding probability is in the region of .04 and cannot be considered significant. The conclusion to be drawn from this is that — above an extrapolated basal value of about 6 mg of urinary phosphorus corresponding to the consumption of totally phosphorus-free food — the quantities which are consumed with the food are excreted in the urine practically quantitatively, when the source of phosphorus is ammonium phosphate and the ingestion of this element does not exceed the limit established in the present investigation. Furthermore, it follows from what has already been said about the rule of the oblique-angled hyperbola that — at still higher levels of phosphorus intake — the regression of urinary phosphorus upon this intake may approach an asymptote parallel to the abscissa, the numerical value of which may be put equal to the constant quantity of phosphorus, absorbed above the proposed limit.

Summary.

(1) Seven adult albino rats of male sex were given a basal diet very low in calcium, phosphorus and fat soluble vitamins and containing about 9.5 per cent tri-stearine, to which different quantities of ammonium phosphate ($(H_4N)_2HPO_4$) had been added. The experiment lasted for 10 days and the intake of calcium and phosphorus with the diet, as well as their excretion in feces and urine were measured for the last five days.

(2) It was found that the variation between the animals in urinary and fecal phosphorus was wide, whereas the variation in urinary and fecal calcium was more narrow.

(3) Various covariations arising from the study have been discussed and analysed numerically:

(a) The reason for the variation in the fecal output of calcium cannot be settled. Not one of the concomitant factors recorded, not even the varying consumption of phosphorus, can be considered responsible for this variation.

(b) The reason for the very wide variation in urinary phosphorus has been found to be the concomitant variation in the intake of phosphorus, and this alone. The amounts of phosphorus consumed are excreted practically quantitatively by the kidneys. In spite of this, the content of calcium in the different samples of urine is rather uniform and not related to their varying content of phosphorus.

(c) As to the variation in the fecal output of phosphorus, the possibility has been discussed that the regression of this output upon the phosphorus intake follows an oblique-angled hyperbola.

(d) The excretion of urinary calcium accounted for about 2.5 per cent only of the total excretion of this element.

Sammanfattning.

Fosfor- och calcium-omsättningen hos fullväxta råttor med ammoniumfosfat som enda fosforkälla och på calcium-fattig näring.

Denna undersökning avhandlar fosfors och calciums omsättning hos fullväxta djur (vita laboratorierättor). Dess uppgift är tvåfaldig: dels att lämna en så fullständig redovisning som möjligt för inkomst och utgifter av dessa grundämnen, dels att utröna de faktorer, som bestämma hur stor del av dem bortgår från kroppen genom tarmen och genom njurarna.

Sasom fosforkälla användes ammoniumfosfatet $(H_4N)_2HPO_4$, som utfodrades i olika mängd åt olika djur, inblandat i försöksnäringen. Av annan fosfor gavs endast det, som fanns kvar i de enskilda näringsbeståndsdelarna efter omsorgsfull rensning. Detsamma gäller calcium. Den mängd av calcium, som förtärdes av djuren per dygn, uppgick till ett värde, som

motsvarar 0,7 gram per 75 kg kroppsvikt. Det är något mer än vad är brukligt för en stor del av vart lands invånare. Den ogynnsamma inverkan av denna ringa calciumförtäring skulle ytterligare förvärras av att försöksdjuren erhöllo en fett-art med mättad fettsyra och hög smältpunkt, nämligen tristearin. Djurens tillgång till D-vitamin var praktiskt taget ingen, något som också torde överensstämma med förhållandet hos de flesta fullväxta människor i vårt land under vintern.

Undersökningens viktigaste resultat äro följande:

1) Försöksdjuren ledo väldiga förluster av calcium med tarmuttömningen, cirka 6 gånger mer än vad samtidigt förtärdes. Dessa förluster, som väsentligen bero på näringens stearin, kunde icke minskas genom att öka fosforförtäringen.

2) Urinens calciummängd var obetydllig och utgjorde blott 2,5 procent av den totala calciumutsöndringen. Den var ganska likartad hos de olika djuren och dessutom oberoende av alla experimentella faktorer.

3) Tarmuttömningens fosformängd var måttlig och ganska likartad hos de olika djuren. Något beroende av andra faktorer kunde icke med säkerhet påvisas, möjligen med undantag av tarmuttömningens calciummängd. Sannolikt resorberades av tarmen all den fosfat-fosfor, som förtärdes, i det att tarmuttömningens ganska konstanta fosforinnehåll torde härstamma från kroppen. Dock tyder undersökningen på att det finns en övre gräns för denna resorption, över vilken den antager ett konstant värde. Då härstammar en stigande del av tarmuttömningens fosfor från näringens fosfor.

4) Den mängd fosfor, som bortgår med försöksdjurens urin, visade sig troget följa den fosformängd, som förtärdes. Dock bortgick mera fosfor med urinen än den motsvarande förtärda mängden. Liksom beträffande calcium ledo alltså djuren fosforförluster, vilka icke kunde minskas, hur mycket ammonium-fosfat-fosfor än förtärdes. — För calcium skedde dessa förluster huvudsakligen med tarmuttömningen, för fosfor huvudsakligen med urinen.

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fälle beobachtet. Nach wiederholter Anwendung entwickelt sich ein Block mit Zeichen der Insuffizienz.

4. Am Herz-Lungen-Präparat (Hund) ruft L. in Gaben zwischen 5 und 10 mg allmählich eine geringgradige Erhöhung des arteriellen und Senkung des venösen Blutdrucks hervor. Eine einmalige größere oder mehrere kleinere Dosen verursachen Arrhythmie, Senkung des arteriellen Blutdrucks und diastolischen Herzstillstand.

5. Bei sämtlichen Tieren wurde die Arbeit des insuffizient gewordenen Herzens durch L. vorübergehend gebessert.

6. Das Durchströmungsvolumen der hinteren Extremitäten der Ratte wird durch 10 mg L. durchschnittlich um 33% des normalen Wertes verkleinert. Der Ausgangswert wird trotz Auswaschen mit frischer Ringerlösung auch nach 20 und mehr min nicht mehr erreicht.

7. An Hunden steigern Dosen von 5 und 15 mg L. innerhalb von 15 sec den Blutdruck durchschnittlich etwa um 12 bzw. 29%. Dabei wird eine Bradykardie und nach größeren Dosen eine Arrythmie beobachtet. Nach Durchschneidung beider Vagus-Nerven stellt bei unveränderter Pulszahl eine merkliche Steige des Blutdrucks ein.

8. Die Blutdrucksteigerung nach 5 mg L. beträg Katzen etwa 25% der Norm. An dekapitierten T stieg der Blutdruck bis zu 50% an.

9. Mäßigen Dosen von Ergotamintartrat (Gyne WZ), 2-Benzyl-4,5-imidazolin-hydrochlorid (Pr WZ) und N-(2'-Diäthyl-aminoäthyl)-phenoth (Diparcol, WZ) sowie die Unterbindung der N nierengefäße beeinflussen nicht die pressorische kung des L. bei Hunden.

10. Die blutdruckerhöhenden Wirkungen des L. weder abhängig vom adrenergischen Mechanis noch von vegetativen Synapsen und vom über Halsmark gelegenen Zentralnervensystem. Dafür lediglich die direkten Wirkungen auf die peripheren Gefäße und die positiv inotrope Herzwirkung verantwortlich.

11. Die pharmakodynamischen Effekte des L. haben mit denen der *Digitalis* eine gewisse Ähnlichkeit.

Summary:

Cardiac and Vascular Action of Luteolin in Animals

1. The cardiac and vascular action of the flavon derivative Luteolin was studied in various animals.

2. In the isolated frog's heart concentrations of 5×10^{-4} Luteolin reduce the diastolic and, somewhat less, the systolic amplitude. It also reduces the heartbeat frequency and the cardiac output. The systolic amplitude may in some cases be increased. In cardiac insufficiency initial temporary increase of the diastolic amplitude is observed. After some minutes arrhythmia is observed. Within 20 minutes, the performance of the dilated heart ceases in consequence of complete block.

3. In the isolated guinea-pig's heart 2×10^{-4} concentrations of luteolin increase the systolic and, somewhat less, the diastolic actions simultaneous with increase of frequency. No direct influence on the blood flow volume in coronary vessels was observed. Following repeated application, heart block with clinical signs of insufficiency results.

4. In heart-lung-preparations (dogs) 5—10 mgs luteolin gradually induce slight increase of the arterial and lowering of the venous blood-pressure. A single large or repeated small doses induce arrhythmia, lowering of arterial blood-pressure, and diastolic cardiac arrest.

5. In all animals temporary increase of heart performance in cardiac insufficiency was observed.

6. 10 mgs luteolin lower the blood flow in rat's hind extremities by an average of 33%. The original value is not re-attained even after 20 minutes or more, even after perfusion with freshly prepared Ringer solution.

7. In dogs, doses of 5 or 15 mgs luteolin will increase the blood pressure within 15 sec. by an average of 12 or 29%. Simultaneously bradycardia and, following large doses, arrhythmia is observed. After bilateral vagotomy marked increase of blood-pressure occurs, the puls frequency remaining unchanged.

8. In cats 5 mgs luteolin raise the blood-pressure about 25% above normal. In decapitated animals the blood-pressure is raised up to 50%.

9. No change of the pressor effect of luteolin in dogs is observed following moderate doses of ergotamine tartrate (trade name: Gynergen), 2-benzyl-4,5-imidazoline-hydrochloride (trade name: Priscol) and, n-(2'-diethyl-aminoethyl)-phenothiazine (trade name: Diparcol) or following ligature of suprarenal vessels.

10. The pressor action of luteolin is independent of the adrenergic mechanism, autonomic nervous synapses, and of the CNS above the cervical level. The pressor effects are solely due to direct action on the peripheric vessels and positive inotropic cardiac action.

11. The pharmacodynamic effects of luteolin are somewhat similar to those of *Digitalis*.

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KURZE MITTEILUNGEN

1012

Aus dem Institut für Pharmakologie, Toxikologie und Pharmazie der Tierärztlichen Fakultät der Universität München; Direktor: Professor Dr. med. K. Zipf

Zur Toxikologie der Polyphosphate

Von Professor Dr. med. K. Zipf

Polyphosphate, vor allem mit kettenförmiger Struktur, dienen vielfach zur Verbesserung der Qualität, zur Erleichterung der technischen Verarbeitung und zur Erhöhung der Haltbarkeit von Nahrungsmitteln. Ihr jahrzehntelanger Gebrauch, gleichsam ein Dauerexperiment im großen, deutet zwar schon auf die absolute Unschädlichkeit der in Lebensmitteln zur Anwendung kommenden Polyphosphat-Mengen hin. Auch das physiologische Vorkommen von Polyphosphaten [1—4] in tierischen und pflanzlichen Zellen und die relativ rasche Aufspaltung der meisten Polyphosphate zu Orthophosphat im menschlichen und tierischen Magen-Darm-Kanal [5, 6] sprechen nicht gerade für eine hohe Toxizität. Trotzdem steht die Erörterung der „Verträglichkeit" der Polyphosphate nach wie vor im Vordergrund des Interesses. Ein Beitrag zu dieser Frage sind Bestimmungen der akuten Toxizität einer Reihe von definierten „Phosphaten" im Rahmen pharmakologischer Untersuchungen mit verschiedener Zielsetzung.

Bestimmt wurde die mittlere letale Dosis (DL_{50}) für weiße Mäuse bei einmaliger peroraler, subkutaner und intravenöser Applikation der „Phosphate" in neutraler wäßriger Lösung. Als physiologische Vergleichssubstanz diente Natriumchlorid.

Aus der Tabelle ergibt sich, daß sämtliche untersuchten Phosphate bei peroraler Zufuhr nur sehr wenig toxisch sind. Während Carbamidorthophosphat und Kochsalz etwa gleich giftig sind, wirken Natriumhexametaphosphat und cyclisches Trinatriumtriometaphosphat wesentlich weniger toxisch als Natriumchlorid. Aber auch die anderen Verbindungen — Hexanatriumtetrapolyphosphat, Tetranatriumpyrophosphat, Dinatrium-

Phos

Tabelle: Mittlere letale Dosis (DL_{50}) in Gramm pro kg Maus

	per os	subkutan	intravenös
Carbamido-orthophosphat $CO(NH_2)_2 \cdot H_3PO_4$	5,84	4,00	0,683
Hexanatriumtetrapolyphosphat $Na_6P_4O_{10}$	5,92	0,873	
Tetranatriumpyrophosphat $Na_4P_2O_7$	2,98	0,40	0,069
Natriumhexametaphosphat $(NaPO_3)_6$	7,23	1,70	0,062
Dinatriumpyrophosphat $Na_2H_2P_2O_7$	2,63	0,48	0,059
Natriumtripolyphosphat $Na_5P_3O_{10}$	3,21	0,90	0,071
cyclisches Trinatriummetaphosphat	10,30	5,94	1,163
Natriumchlorid	3,99	3,00	0,645

pyrophosphat und Natriumtripolyphosphat — zeigen nur eine sehr geringe Toxizität. Bei peroraler Zufuhr weisen die untersuchten „Phosphate" demnach keine spezifische, sondern nur eine rein osmotisch bedingte Salzwirkung auf. Auch bei sc. und iv. Injektion ist die Toxizität, verglichen mit anderen Wirkstoffen, relativ gering.

Carbamidoorthophosphat und cyclisches Trinatriummetaphosphat sind weniger giftig als auf denselben Wegen zugeführtes Natriumchlorid. Lediglich Tetranatriumpyrophosphat, Dinatriumpyrophosphat, Natriumhexametaphosphat und Natriumtripolyphosphat besitzen bei sc. und iv. Injektion eine Toxizität, die auf pharmakologische Eigenwirkung schließen läßt. Für die perorale Verträglichkeit ist diese jedoch nicht von Bedeutung, da die genannten Phosphate enteral überhaupt nicht oder fast vollständig als Orthophosphat resorbiert werden [5–7].

Zusammenfassung:

Im Tierversuch sind Polyphosphate bei peroraler, subkutaner und intravenöser Zufuhr nur wenig toxisch. Bei peroraler Zufuhr beruht ihre Wirkung auf einem osmotischen Effekt.

Summary:

Studies on the Toxicology of Polyphosphates

In animal experiments, polyphosphates showed negligible toxicity in peroral, subcutaneous, or intravenous application. In peroral application the effect is due to osmotic action.

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Aus der Hautklinik der Medizinischen Akademie Düsseldorf; Direktor: Professor Dr. med. H. Th. Schreus

Über Lichtschutzmittel

4. Mitteilung [1]: Schichtmessung mit ^{131}Jod

Herrn Professor Dr. J. Vonkennel zum 60. Geburtstag gewidmet

Von Dr. H. Ippen und H. Betzler

Präparate, welche die menschliche Haut vor übermäßigem Lichteinfall schützen sollen, müssen lichtabsorbierende Eigenschaften besitzen. Diese werden durch das Absorptionsmaximum des Wirkstoffes einerseits und durch die Dicke der auf der Haut gebildeten Schicht andrerseits bestimmt. Deshalb stellt das Absorptionsspektrum zunächst das wichtigste Kriterium für die Auswahl geeigneter Filtersubstanzen dar. Mit dessen Hilfe kann, besonders durch Berechnung der „kritischen Schichtdicke" [4], mit ziemlicher Sicherheit der Wert einer solchen Filtersubstanz ermittelt werden. Dieses Berechnungsverfahren zeigt aber auch eindeutig die Wichtigkeit der Kenntnis der jeweils zu erwartenden Schichtdicke. Denn die Höhe des Zusatzes an Filtersubstanz muß diese weitgehend berücksichtigen, wenn das Fertigpräparat nicht zu schwach (also Sonnenbrand) oder zu stark (also Ausbleiben der erwünschten Pigmentierung) lichtabsorbierend sein soll.

Die bisherigen Verfahren zur Bestimmung der Schichtdicke von Externa auf der Haut bestanden meist in der Verteilung einer bestimmten Menge auf der Haut und Berechnung der Schichtdicke aus der Größe der mit den Präparat bedeckten Fläche. So wurden Werte gefunden, die sich in der Größenordnung von 5 bis 20 μ bewegten [2]. Ganz abgesehen von der nicht sehr hohen Genauigkeit dieses Verfahrens kann man hiermit immer nur Mittelwerte erhalten und nicht etwa auch die minimale und maximale Schichtdicke bestimmen. Ein noch größerer Nachteil dieser Methode ist darin zu sehen, daß sie für ein Gruppe von Präparaten, die „Aerosole", nicht anwendbar ist. Würde man diese Präparate in abgemessener Menge ohne Treibmittel verreiben, so blieben niedrigere Schichtdicken, wie sie beim üblichen Versprayen durchaus auftreten können, völlig unberücksichtigt. Das führt zu einer zu niedrigen Bemessung des Filteranteiles und damit zu einer relativ schwachen Wirkung der „Aerosol"-Präparate [3].

Wir versuchten deshalb, durch eine grundsätzlich andere Methode die Nachteile des beschriebenen Verfahrens zu vermeiden. Wir markierten einige übliche Salbengrundlagen durch Zusatz ^{131}Jod-haltiger Dijodölsäure, die uns von der Firma Bayer, Leverkusen, freundlicherweise hergestellt und überlassen wurde. Nachdem in Vorversuchen durch Variation der Schichtdicke und der spezifischen Aktivität zuverlässige Eichkurven ermittelt worden waren [3], wurde die Radioaktivität über den eingeriebenen Hautpartien gemessen. Es wurden an den jeweils etwa 100 cm² großen Flächen an verschiedenen Körperpartien mehrere Messungen vorgenommen, um die Schwankungen bei einer Auftragung zu erfassen.

Bei jeweils mindestens 60 Messungen wurden die in Tab. 1 aufgeführten Schichtdicken gefunden (alle Werte in μ).

Tabelle 1

	Durchschnitt	Tiefstwert	Höchstwert
Gelbe Vaseline	4—12	3	23 (33)
Eucerin® c. aqua	3—8	1	22
Paraffinöl	2—6	1	15
Glycerincreme (fettfrei)	7—19	4	21
alkoholische Lösung (10% Isopropylmyristat in Isopropylalkohol)	0,25—1,25	0,25	1,25
„Aerosol" (Isopropylalkohol mit Isopropylmyristat zu gleichen Teilen, dazu übl. Treibmittel)	1—9	unter 1	21

Wie diese Werte zeigen, liegen die Schichtdicken bei den geprüften Grundlagen in der Mehrzahl unter 10 μ, wobei noch erwähnt werden muß, daß außer bei Vaseline und Glycerincreme die am häufigsten gemessene Dicke unter 3 μ lag.

Zusammenfassung:

Verschiedene Externa, darunter ein Aerosol, wurden durch Zusatz von ^{131}J-Dijodölsäure radioaktiv gemacht und nach entsprechender Eichung die Schichtdicke dieser Präparate nach Auftragen auf die Haut bestimmt. Für alle Zubereitungen wurden Mittelwerte unter 10 μ erhalten. Außer bei Vaseline und Glycerincreme wurden am häufigsten Werte unter 5 μ gemessen.

Summary:

Light Protection Ointments

Several external remedies, among them an aerosol have been made radioactive by an addition of ^{131}J-diiodoleic acid. Following calibration, the thickness of the layers applied to the skin was measured. The mean values were found below 10 μ in all preparations. Except in vaseline and glycerine cream, the values most frequently measured were below 5 μ.

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Virchows Archiv, Bd. 324, S. 531—542 (1954).

Pathol. Anat. Physiol

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(Direktor: Prof. Dr. I. Gy. Fazekas).

S2

Vergrößerung der Nebenschilddrüsen durch einfache acidotische Verbindungen.

Von

I. Gy. Fazekas.

Mit 3 Textabbildungen.

(Eingegangen am 7. Juli 1953.)

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Über Vergrößerung menschlicher Nebenschilddrüsen wurde zuerst von Erdheim (1903), später von Claude und Baudouin, Reinhard und Creuzfeld, Ballet und Lavastine, Josephson, Erdheim, Cushing und Davidoff, Hoff u. a. in Verbindung mit basophilen Adenomen des Hypophysenvorderlappens (HVL) sowie von Lloyd in Verbindung mit Hauptzellenadenomen der Hypophyse berichtet. Recklinghausen beschrieb 1904 das Krankheitsbild der Osteitis fibrosa generalisata, die sich unter dem Einfluß der adenomisch oder hyperplastisch bedingten gesteigerten Nebenschilddrüsenfunktion ausbildet. Der Zusammenhang zwischen dieser Knochen- und Nebenschilddrüsenveränderung bzw. -hyperfunktion wurde zuerst von Mandl (1926) und später von anderen dadurch bewiesen, daß die Kranken nach operativer Entfernung ihrer adenomischen oder hyperplastischen Nebenschilddrüsen gesund wurden. Die sog. *sekundäre Hyperplasie* der Nebenschilddrüsen kann als Kompensationsreaktion bei gesteigertem Parathormonbedürfnis des Organismus vorkommen, so z. B. bei Knochenmetastasen eines Carcinoms, bei einem Myeloma multiplex, einer Rachitis, Osteomalacie, Cushingschen Krankheit, akuten Nierenerkrankung (Cameron, Verzár), bei Gravidität (Árvay), bei partieller oder totaler Nierenexstirpation sowie bei einem Calcium- und Phosphorgehaltanwuchs der Speisecordnung (Courxot).

Anselmino, Hoffmann und Herold beobachteten nach mehrtägiger Verabreichung von HVL-Extrakt an Ratten, später auch an anderen Tieren, Hertz und Kranes an Kaninchen, Ham und Hais an Hunden Vergrößerungen der Nebenschilddrüse. Auf Grund dieser Beobachtungen nahmen sie an, daß der HVL ein die Nebenschilddrüsenfunktion stimulierendes Hormon bildet. Damit stehen in vollem Einklang die Beobachtungen, nach denen Smith an Ratten, Köster, Houssay und Biasotti, sowie Houssay und Sammartino, aber auch Vernetti an Hunden nach Exstirpation der Hypophyse eine Atrophie und degenerative Atrophie der Nebenschilddrüsen vorfinden konnten. Livon und Peyron sowie Aschner sahen hingegen an Hunden, später Collip an Ratten keine Schilddrüsenatrophie nach Entfernung der Hypophyse. Thompson und Cushing konnten an Hunden, Cittaneo an Kaninchen durch Hypophysenextrakt keine Vergrößerung der

Sulf.

Nebenschilddrüsen erzielen. 1949 beobachtete Törnblom an Kaninchen nach Verabreichung von großer Menge Natrium- und Ammoniumphosphat bei einer 12wöchigen calciumarmen Speisefolge eine hochgradige Vergrößerung der Nebenschilddrüse.

Die bisherigen Untersuchungen stellten klar, daß die gesteigerte oder verminderte Funktion der Nebenschilddrüsen durch die im Calcium- und Phosphorstoffwechsel des Organismus verursachten tiefgreifenden Veränderungen verschiedene pathologische Zustände hervorruft. Es wäre also wünschenswert, die Funktion der Nebenschilddrüse auf chemischem Wege so beeinflussen zu können, daß sie durch ihre verminderte Funktion bedingten Erkrankungen durch die Wiederherstellung der Funktion zu beheben wären.

Im Laufe früherer Untersuchungen (1938—1950) wurde schon festgestellt (Fazekas), daß durch dauerhafte Verabreichung zahlreicher acidotisch wirkender Verbindungen in Kaninchen, Gänsen, Schweinen und Ziegen Hypertrophie und gesteigerte Funktion der Nebennierenrinde, Vergrößerung der Ovarien und des Uterus, Follikelreifung und -blutung, Gelbkörperbildung, cyclische Veränderungen an der Schleimhaut der Uterushörner, gesteigertes Fettwerden, Vergrößerung der Brüste und Milchsekretion sowie Vergrößerung der Hypophyse und Vermehrung der basophilen Zellen des HVL erzielt werden konnten. — Auf die Möglichkeit der chemischen Beeinflussung der Nebenschilddrüsen sollen folgende Untersuchungen hinweisen.

Zur Behandlung der Tiere wurden die in der Tabelle angeführten Verbindungen verwendet. Die Nebenschilddrüsen von 362 behandelten Kaninchen (darunter befanden sich auch Tiere aus vorhergehenden Versuchen) wurden untersucht, Größe, Gewicht und histologischer Befund mit den entsprechenden Angaben der Kontrolltiere verglichen. Als Kontrollen dienten 50 gleichaltrige und -artige Kaninchen mit gleichem Gewicht. — Aus der Übersichtstabelle geht es hervor, daß die Nebenschilddrüsen der unbehandelten Kontrolltiere 6—14 mg, im Mittelwert 9,2 mg wogen, die der behandelten Kaninchen hingegen an Gewicht erheblich zunahmen.

1. Mit *Ammoniumhydroxid* wurden 96 weibliche und 64 männliche Tiere 4—5—16 Monate lang behandelt. Jedes Tier bekam 2täglich 50 bis 80 cm³ $^1/_2$%iges NH_4OH durch Magensonde in allmählich zunehmenden Dosen. Nach einer 3wöchigen Behandlung folgte immer eine 1wöchige behandlungsfreie Periode. Das Körpergewicht der Tiere nahm im Laufe der Behandlung allmählich zu und betrug bei der Tötung mit Luftembolie 3100—4400 g. — Die Nebenschilddrüsen wogen 17—39 mg, im Mittelwert 26 mg, was im Verhältnis zum Gewicht der Nebenschilddrüsen der Kontrollen um 16,8 mg, d. h. um 182,6% mehr war. Auf

Tabelle 1. *Gewichtsangaben der Nebenschilddrüsen bei behandelten Kaninchen und Kontrollen.*

		Zahl der Tiere	Gewicht der Nebenschilddrüsen in mg			Gewichtszunahme der Nebenschilddrüsen im Mittelwert		S. D.
			minimal	maximal	Mittelwert	%	mg	
	Kontrollen	50	6	14	9,2	—	—	—
1	Ammoniumhydroxyd	160	17,0	39,0	26,0	16,8	182,6	10,84
2	Ammoniumchlorid	80	16,5	57,5	34,5	25,3	274,6	9,03
3	Ammoniumsulfat	10	15,0	33,0	27,5	18,3	198,9	7,04
4	Ammoniumcarbonat	14	15,0	40,0	25,0	15,8	171,9	6,86
5	Natriumammoniumphosphat	14	19,0	51,0	32,5	23,3	254,3	6,50
6	Ammoniumacetat	12	18,0	49,0	34,5	25,3	274,6	9,73
7	Ammoniumlactat	10	17,5	52,0	29,5	20,3	219,5	6,15
8	Caclciumchlorid	10	21,0	54,0	32,5	23,3	251,0	7,76
9	Acidum hydrochloricum	10	16,0	48,0	28,5	19,3	210,8	6,66
10	Acidum lacticum	10	22,5	43,0	29,5	20,3	219,5	5,27
11	Acidum aceticum	12	20,0	36,0	28,6	19,3	209,7	12,12
12	Natriumdihydrophosphat	10	24,0	50,0	36,5	27,3	294,5	10,72
13	Ammoniumhydrophosphat	10	16,5	43,0	30,5	21,3	234,8	7,10

Grund der statistischen Rechnungen betrug die signifikante Differenz 10,84, die Gewichtszunahme der Nebenschilddrüsen ist also spezifisch.

2. Mit *Ammoniumchlorid* wurden 50 weibliche und 30 männliche Kaninchen 3—5—16 Monate behandelt. Zweitäglich bekamen die Tiere 0,1—0,2 g/kg NH_4Cl in 100—150 cm³ Trinkwasser gelöst, in allmählich zunehmenden Dosen. Nach 3wöchiger Behandlung wurde immer eine Behandlungspause von 1 Woche eingeschaltet. Das Gewicht der Tiere nahm während der Behandlungsdauer allmählich zu und betrug bei der Tötung mit Luftembolie 3500—4500 g. Das Gewicht der Nebenschilddrüsen betrug 16,5—57,5 mg, im Mittelwert 34,5 mg, das ist um 25,3 mg mehr als bei den Kontrollen, das einer 274,6%igen Gewichtszunahme entspricht. Die Signifikanz wurde für 9,03 gefunden.

3. *Ammoniumsulfat* wurde 10 weiblichen Kaninchen 5—16 Monate auf gleiche Art und in gleichen Dosen verabreicht. Körpergewicht bei Tötung 4100—4800 g. Gewicht der Nebenschilddrüsen 15—33 mg, im Mittelwert 27,5 mg, um 18,3 mg mehr als bei den Kontrollen, was 198,9%iges Plus bedeutet. Differentia significans: 7,04.

4. Mit *Ammoniumcarbonat* wurden 14 weibliche Kaninchen 5 bis 16 Monate behandelt. Gleiche Behandlungsmethode. — Körpergewicht bei Tötung: 4000—4700 g. Gewicht der Nebenschilddrüsen 15—40 mg,

im Mittelwert 25 mg, um 15,8 mg mehr, als bei den Kontrollen. Die Gewichtszunahme betrug also 171,9% mit einer Signifikanz von 6,86.

5. *Natriumammoniumphosphat* wurde gleichfalls 14 weiblichen Kaninchen 5—14 Monate mit gleicher Methode gegeben. Körpergewicht bei Tötung: 3800—4700 g. Gewicht der Nebenschilddrüsen 19—51 mg, im Mittelwert 32,5 mg, d. h. um 23,3 mg mehr als bei den unbehandelten Kontrolltieren. Das ist eine 254,3%ige Gewichtszunahme mit einer signifikanten Differenz von 6,5.

6. *Ammoniumacetat* wurde 12 weiblichen Kaninchen 5—16 Monate lang verabreicht. Behandlungsmethode wie vorher. Körpergewicht bei Tötung: 3500—4800 g. Die Nebenschilddrüsen wogen 18—49 mg, im Mittelwert 34,5 mg, um 25,3 mg mehr als bei den normalen Tieren: ein Gewichtsplus von 274,6%. Differentia significans 9,73.

7. Mit *Ammoniumlactat* wurden 10 weibliche Kaninchen während 5—16 Monate behandelt. Gleiche Behandlungsmethode. Körpergewicht bei Tötung: 3350—4700 g. Gewicht der Nebenschilddrüsen 17,5 bis 52 mg, im Mittelwert 29,5 mg, das ist um 20,3 mg mehr als bei den Kontrollen, das eine 219,5%ige Gewichtszunahme bedeutet. Signifikanz: 6,15.

8. Mit *Calciumchlorid* wurden 10 weibliche Kaninchen 5—16 Monate gleicherweise behandelt. Körpergewicht bei Tötung: 3700—5050 g. Gewicht der Nebenschilddrüsen 21—54 mg, im Mittelwert 32,5 mg, d. h. um 23,3 mg (251%) mehr als bei den Kontrollen. Differentia significans: 7,76.

9. Mit *Acidum hydrochloricum* wurden 10 weibliche Kaninchen 5 bis 16 Monate lang behandelt. Diese Tiere bekamen 2täglich 0,5—1,0 cm³ 25%iges HCl in 100—150 cm³ Trinkwasser, in allmählich zunehmenden Dosen. — Auch hier folgte nach einer Behandlung von 3 Wochen eine 1wöchige behandlungsfreie Pause. Körpergewicht bei Tötung der Tiere 3500—4500 g. Gewicht der Nebenschilddrüsen 16—48 mg, im Mittelwert 28,5 mg, um 19,3 mg mehr als bei den normalen Tieren. Das bedeutet ein Gewichtsplus von 210,8%, mit einer Signifikanz von 6,66.

10. *Acidum lacticum* wurde 10 weiblichen Kaninchen 5—16 Monate hindurch gegeben. Den Tieren wurde 2täglich in zunehmenden Dosen 0,2—0,5 cm³/kg Wirkungsstoff in 100—150 cm³ Trinkwasser verabreicht. Nach 3 Wochen Behandlung folgte 1 Woche Pause. Bei Tötung betrug das Körpergewicht 3700—4800 g. — Gewicht der Nebenschilddrüsen 22,5—43 mg, im Mittelwert 29,5 mg, also um 20,3 mg, d. h. um 219,5% mehr als bei den Kontrollen. Signifikante Differenz 5,27.

11. Mit *Acidum aceticum* wurden 12 weibliche Kaninchen 5—16 Monate behandelt. Die Tiere bekamen 2tägig in zunehmenden Dosen 0,2 bis 0,5 cm³/kg konzentrierte Essigsäure in 100—150 cm³ Trinkwasser. Körpergewicht bei Tötung 3600—4600 g. Gewicht der Nebenschild-

drüsen 20—36 mg, im Mittelwert 28,6 mg, d. h. um 19,3 mg mehr als bei den normalen Kontrolltieren, was einer Zunahme von 209,7% entspricht. Differentia significans 12,12.

12. Mit *Natriumdihydrophosphat* wurden 10 weibliche Kaninchen 5—16 Monate lang behandelt. Diese Tiere bekamen aber 0,3 bis 0,7 g/kg Wirkungsstoff 2täglich in zunehmenden Dosen in 100 bis 150 cm³ Trinkwasser. Körpergewicht bei Tötung: 4000—4500 g. Gewicht der Nebenschilddrüsen betrug 24—50 mg, im Mittelwert 36,5 mg, also um 27,3 mg mehr als bei den Kontrollen. Das bedeutet eine 294,5%ige Gewichtszunahme mit einer Signifikanz von 10,72.

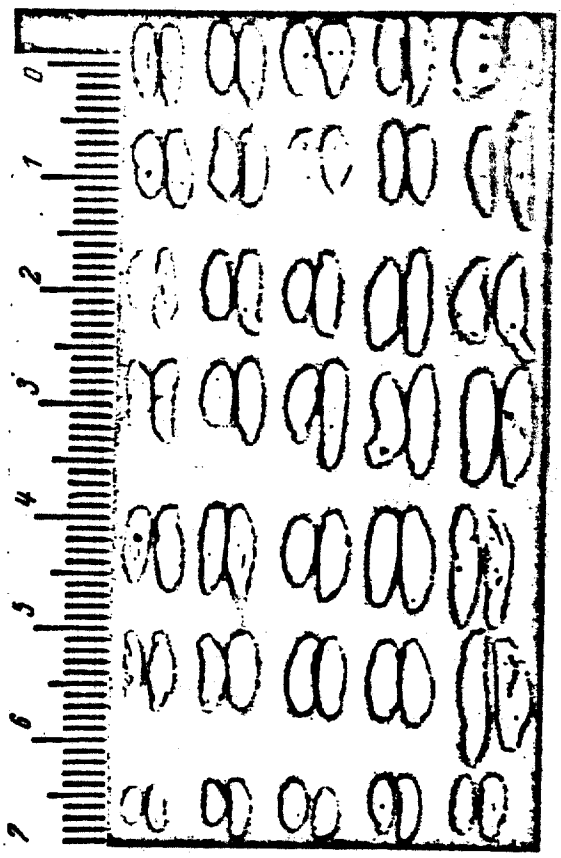

Abb. 1. Vergrößerung der Nebenschilddrüsen bei 5 Monate lang mit verschiedenen Verbindungen behandelten Kaninchen. — I. Reihe: Ammoniumchloridbehandlung: 16,5—18—24,5—26—57,5 mg. — II. Reihe: Ammoniumsulfatbehandlung: 15—16—17—20—27,5 mg. — III. Reihe: Ammoniumcarbonatbehandlung: 15—18—20—27,5 bis 40 mg. — IV. Reihe: Na-Ammoniumphosphatbehandlung: 19—20,5—25—37,5—51 mg. — V. Reihe: Ammoniumacetatbehandlung: 18—22,5—24—39—43 mg. — VI. Reihe: Ammoniumlactatbehandlung: 17,5—20—21—30—52 mg. — VII. Reihe: Kontrollen ohne Behandlung: 6—7—8 bis 9,5—10 mg.

13. *Ammoniumhydrophosphat* wurden 10 weiblichen Kaninchen 5 bis 16 Monate hindurch verabreicht. Ein Tier bekam 0,3 bis 0,7 g/kg Verbindung 2täglich in zunehmenden Dosen in 100 bis 150 cm³ Trinkwasser. Körpergewicht bei Tötung: 3900—4800 g. Gewicht der Nebenschilddrüsen 16,5—43 mg, im Mittelwert 30,5 mg, d. h. um 21,3 mg mehr (234,8%) als bei den Kontrollen. Signifikante Differenz: 7,1.

Mit Hinsicht auf die angeführten Angaben kann behauptet werden, daß die Nebenschilddrüsen unter Wirkung der verwendeten Verbindungen

an Gewicht erheblich zunehmen, und zwar maximal bei den mit Natriumdihydrophosphat (294,5%), Ammoniumchlorid bzw. Ammoniumacetat (274,6%), minimal bei den mit Ammoniumcarbonat (171,9%) und Ammoniumhydroxid (182,6%) behandelten Tieren. — Auf Grund der statistischen Berechnungsergebnisse kann die Gewichtszunahme der Nebenschilddrüsen einstimmig nur spezifisch gehalten

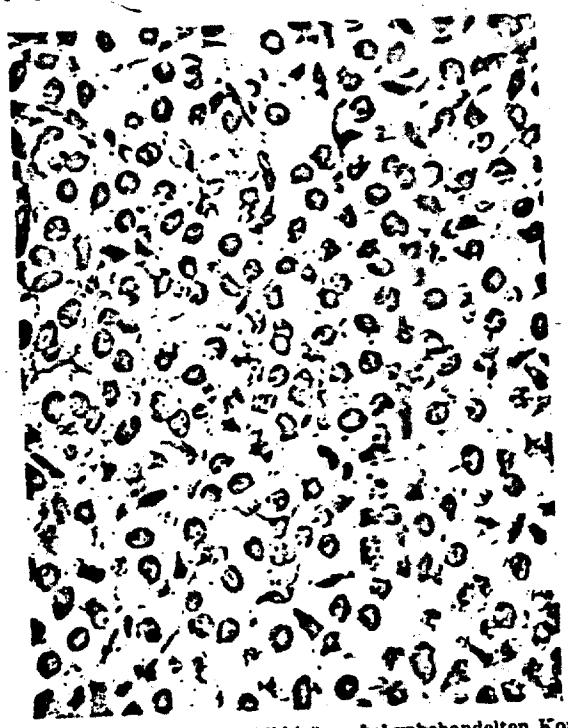

Abb. 2. Histologisches Bild der Nebenschilddrüsen bei unbehandelten Kontrollkaninchen. In überwiegender Mehrheit bilden dunkle Hauptzellen die Drüsensubstanz. Die hellen Hauptzellen sind in verhältnismäßig kleiner Zahl und von normaler Größe. — Hämatoxylin-Eosinfärbung. 1000fache Vergrößerung.

werden. — Die Gewichtszunahme ging mit einer auffallenden Vergrößerung der Nebenschilddrüsen an Volumen bzw. Dimensionen einher (Abb. 1).

Im Laufe der *histologischen Untersuchung* wurde beobachtet, daß die Gefäße der Nebenschilddrüsen bei den *unbehandelten* Kontrollen im allgemeinen mittelmäßig blutreich und mittelmäßig weit waren. Die Hauptmasse der Drüsen bildeten die sog. *dunklen Hauptzellen*, an den Drüsenrandteilen konnten zerstreut *oxyphile Zellen* vorgefunden werden (Abb. 2). — In den Nebenschilddrüsen der *behandelten* Kaninchen hingegen waren die größeren Gefäße, aber auch die Capillaren, schon nach 3monatiger Behandlung weit und blutvoll, die Hauptmasse der

Drüsen bestand aus vergrößerten und auch an Zahl vermehrten sog. *hellen Hauptzellen*. Das Plasma dieser Zelltypen war vergrößert, hell, oft befanden sich darin Vacuolen. Ihr Kern war gleichfalls groß und färbte sich hell. In mehreren solchen Zellen waren Kernteilungen zu sehen. Dunkle Hauptzellen wurden in den Nebenschilddrüsen der behandelten Tiere nur in verhältnismäßig geringer Zahl vorgefunden.

Abb. 3. Histologisches Bild der vergrößerten Nebenschilddrüsen eines 3 Monate lang mit NH_4Cl behandelten Kaninchens. Die Drüsensubstanz ist besonders von hellen Hauptzellen mit vergrößertem Plasma gebildet. — Hämatoxylin-Eosinfärbung. 1000fache Vergrößerung.

Die oxyphilen Zellen fehlten fast vollkommen in den 3—5monatigen Fällen, in den Fällen von 16 Monaten waren sie aber schon meistens aufzufinden. Vermehrung des Bindegewebes konnte nicht einmal nach 16monatiger Behandlung in den Nebenschilddrüsen der Tiere beobachtet werden (Abb. 3).

Gleiches histologisches Bild fanden auch ANSELMINO, HOFFMANN und HEROLD bei Ratten, HERTZ und KRANES bei Kaninchen, HOUSSAY bei Hunden und Kaninchen nach Verabreichung wäßrigen HVL-Extraktes. Verfasser betrachten diese Erscheinungen als Folge der auf die Funktion der Nebenschilddrüsen ausgeübten stimulierenden HVL-Wirkung. Nach den Beobachtungen von HOUSSAY konnten schon 5 Tage

nach der Hypophysektomie Atrophie des Zellprotoplasmas, Verminderung der Zellen und Verschwommenheit der Zellgrenze festgestellt werden. Gleichzeitig verschwand die gleichmäßige epitheliale Struktur der Drüsen, und es bildete sich eine trabeculare, bandelartige Anordnung der Zellen aus. Solche Atrophie der Nebenschilddrüsen war besonders bei Hunden auffallend, an denen Hypophysektomie und Pankreasexstirpation gleichzeitig durchgeführt worden sind. Vernetti beobachtete in den atrophischen Nebenschilddrüsen hypophysektomisierter Hunde auch erhebliche Vermehrung des Bindegewebs.

Im Laufe eigener Untersuchungen zeigten sich in den Nebenschilddrüsen der behandelten Kaninchen Blutreichtum, Hypertrophie und Hyperplasie, erhebliche Vermehrung der hellen Hauptzellen und Kernteilungserscheinungen. Auf Grund all dessen könnte auf die gesteigerte Funktion der infolge der verwendeten Behandlung vergrößerten Nebenschilddrüsen geschlossen werden. Darüber werden wir aber eine endgültige Meinung nur auf Grund der an den vergrößerten Nebenschilddrüsen durchgeführten Funktionsuntersuchungen äußern können.

Es ergibt sich nun die Frage, worin besteht der Funktionsmechanismus der beobachteten, behandlungsbedingten Vergrößerung der Nebenschilddrüsen. Da sich unter den 13 verabreichten Verbindungen organische und anorganische, ammoniakalische und nichtammoniakalische Verbindungen sowie Säuren und Laugen befinden, kann nicht daran gedacht werden, daß die beschriebene Vergrößerung der Nebenschilddrüsen durch die spezifische Einwirkung dieser Verbindungen bedingt werden könne. Es muß also, in betreff des Wirkungsmechanismus, nach einer gemeinsamen Ursache gesucht werden, die im Organismus unter dem Einfluß dieser Verbindungen immer auftritt.

Es ist im allgemeinen bekannt, daß die Säuren und sauren Salze das Säurebasengleichgewicht des Organismus in die saure Richtung verschieben (Acidose), weniger bekannt ist es aber, daß auch die Laugen eine Acidose verursachen können. Fazekas wies nach, daß NH_4OH und NaOH — trotz ihrem Laugencharakter — im Organismus eine Acidose hervorrufen. Seine Behauptungen fanden auch von Venulet, Goebel und Tislowitz, Alvall und Geiger sowie von Hazard und Vaille Bestätigung. Die gemeinsame Eigenschaft der verwendeten Verbindungen besteht also darin, daß sie das Säurebasengleichgewicht des Organismus in die acidotische Richtung verschieben. Unserer Ansicht nach wirkt die Verschiebung des Blutchemismus in die Säurerichtung reizend, entweder unmittelbar auf den HVL oder auf dem Weg über das Hypothalamuszentrum der Hypophyse auf die entsprechenden Zellen des HVL und stimuliert sie dadurch zur gesteigerten Funktion. Die gesteigerte Hypophysenfunktion ruft dann die Vergrößerung bzw. die gesteigerte Funktion der Nebenschilddrüse hervor

Dafür spricht unser Befund, daß sich auch die Hypophyse bei solch behandelten Tieren vergrößert und die basophilen Zellen des HVL an Zahl zunehmen. Gleichfalls für die gesteigerte HVL-Funktion sprechen unsere früheren Behauptungen, daß durch die Einwirkung dieser Verbindungen bei Kaninchen Vergrößerung und gesteigerte Funktion der Nebennierenrinde, in den Eierstöcken Follikelreifung und -blutung sowie Gelbkörperbildung hervorgerufen werden.

Auf die Funktionssteigerung der Hypophyse unter acidotischem Einfluß weisen auch folgende Literaturangaben hin: JULESZ fand an infantilen Mäusen eine Follikelreifung nach Einspritzung von Urin von Männern und Frauen an ketogener Diät. SELYE und HERLANT beobachteten, außer Hyperplasie der Nebennierenrinde, gleiche Erscheinungen nach intravenöser Einspritzung von Salzsäure. — HARRIS spritzte, in die 3. Hirnkammer von Kaninchen, eine Cuprumacetatlösung, was durch die Reizung der Hypothalamuszentren eine Ovulation auslöste. Eine chronische Acidose ist meistens bei jenen menschlichen Erkrankungen nachzuweisen, wo laut den Literaturangaben eine Vergrößerung der Nebenschilddrüsen beobachtet wurde; z. B. bei chronischen Nierenerkrankungen, Schwangerschaft, partieller oder totaler Exstirpation der Nieren usw.

TÖRNBLOM beobachtete bei Kaninchen nach Verabreichung von Natrium- und Ammoniumphosphat Vergrößerung der Nebenschilddrüsen, gleichzeitige Abnahme des Blutcalciums und hochgradige Zunahme des Blutphosphors. Die Behandlung wurde von ihm an hypophysektomisierten Tieren wiederholt, danach eine Herabsetzung des Blutcalcium- und -Phosphorgehaltes festgestellt, die Nebenschilddrüsen vergrößerten sich aber nicht. TÖRNBLOM schloß daraus, daß die Vergrößerung der Nebenschilddrüse durch die Hyperphosphatämie verursacht wurde. In anderen Versuchsserien forschte er nach, von was für Hormoneinwirkungen die Veränderung der Phosphorämie abhänge. Er führte an einer Kaninchengruppe Schilddrüsen- und Nebenschilddrüsenentfernung, an der anderen Schilddrüsen-, Nebenschilddrüsen- und Hypophysenexstirpation durch. Bei diesen Tieren war die Hypocalcämie und Hyperphosphatämie kleiner. Wenn aber den schilddrüsen- und nebenschilddrüsenberaubten Tieren Hypophysenextrakt verabreicht wurde, veränderte sich die Hypocalcämie nicht, die Hyperphosphatämie war aber wesentlich höher. Das weist — seiner Meinung nach — darauf hin, daß die Hypophyse die Phosphorämie verstärkt, ohne die Vermittlung der Nebenschilddrüsen in Anspruch genommen zu haben. TÖRNBLOM veranschaulicht den Einfluß der Hypophyse auf die Nebenschilddrüse mit folgendem Schema: Hypophyse—Hyperphosphatämie—Hyperfunktion der Nebenschilddrüse. Nach seiner Auffassung geschieht die Reizung der Nebenschilddrüse nicht unmittelbar durch die Hypophyse, sondern auf dem Weg über einen humoralen Faktor durch die Steigerung des Blutphosphorniveaus.

Das Ergebnis unserer eigenen Versuche scheint dieser Ansicht von TÖRNBLOM zu widersprechen, da wir außer 3 Phosphatarten noch 10 andere phosphorfreie Verbindungen verabreicht haben und danach eine Vergrößerung der Nebenschilddrüse beobachten konnten. In vorigen Untersuchungen wurde behauptet (FAZEKAS), daß der anorganische Blutphosphorgehalt bei Kaninchen durch einmalige Verabreichung einer größeren Dosis von NH_4OH und NaOH erheblich anwächst, das Serumcalcium und -chlor abnimmt, eine Hyperglykämie auftritt, die Alkalireserve des Blutserums absinkt (!) und die Konzentration des Hydro-

genions zunimmt, d. h. eine Acidose hervorgerufen wird. Eine Hyperphosphatämie kann also auch ohne Phosphorzufuhr zustande kommen. Laut unseren Erfahrungen ist der Zuwachs des anorganischen Blut-Phosphor-Gehaltes bei den infolge verschiedenster Ursachen (Vergiftungen, Erkrankungen) auftretenden acidotischen Zuständen fast immer nachzuweisen. Diese Tatsache scheint Törnblom's Auffassung zu unterstützen. Laut den ausgedehnten und gründlichen Untersuchungen von Baló nimmt der anorganische Phosphorgehalt des Blutserums auch im Falle des nachherigen Sauerwerden des Blutes in vitro erheblich zu, ohne hier durch die Hypophyse beeinflußt zu sein. Demnach kann also der Phosphorgehalt des Blutserums auch ohne Hypophyseneinwirkung ansteigen (das organisch gebundene P der roten Blutkörperchen geht in eine anorganische Bindung über und gelangt in das Blutserum). All das weist darauf hin, daß die Steigerung des Blutphosphorgehaltes als Teilerscheinung der Acidose angesehen werden kann und ihr Dasein an sich keinen Beweis außer allem Zweifel dafür bietet, als ob die Vergrößerung der Nebenschilddrüsen bzw. ihre Funktionssteigerung durch die Hyperphosphatämie bedingt wäre.

Auf Grund eigener und anderer Versuchsergebnisse kommt die Vergrößerung bzw. gesteigerte Funktion der Nebenschilddrüse — unserer Ansicht nach — so zustande, daß die durch verschiedene Ursachen bedingte Verschiebung des Blutchemismus in die Säurerichtung entweder unmittelbar oder auf dem Weg über das Hypothalamuszentrum der Hypophyse auf den HVL reizend wirkt, dieses Organ zur gesteigerten Funktion stimuliert, was dann durch die gesteigerte Bildung von Parathyreotrophormon die Vergrößerung der Nebenschilddrüsen bzw. ihre gesteigerte Funktion verursacht.

Vorliegende Untersuchungen dienen mit neuen Angaben darüber daß das innersekretorische Drüsensystem ohne Hormondarreichung bloß durch Zufuhr von einfachen Verbindungen auf chemischem Wege beeinflußt bzw. seine Funktion umgestellt werden kann. — Angesichts der Nebenschilddrüse kann aber Endgültiges nur auf Grund neuer Ergebnisse ausgesprochen werden. Die diesbezüglichen Untersuchungen sind im Gange und hoffentlich können Hypofunktion von Nebenschilddrüsen und auch dadurch bedingte pathologische Zustände behoben werden.

Zusammenfassung.

1. 362 Kaninchen wurden 2täglich 3—5—16 Monate hindurch organische und anorganische Ammoniak- und andere Verbindungen (0,1 bis 0,2 g/kg) verabreicht. (Die gemeinsame Eigenschaft dieser Verbindungen besteht darin, daß sie das Säurebasengleichgewicht des Organismus in die Säurerichtung verschieben.) Das Gewicht, die Größe und das hist-

logische Bild der Nebenschilddrüsen solch behandelter Kaninchen wurde mit den entsprechenden Angaben von 50 unbehandelten gleichaltrigen Kontrollkaninchen von gleicher Art und gleichem Gewicht verglichen.

2. Das minimale Gewicht der Nebenschilddrüsen bei Kontrollen betrug 6 mg, das maximale 14 mg, im Mittelwert wurden 9,2 mg vorgefunden.

3. Im Gegensatz betrug das minimale Gewicht der Nebenschilddrüsen der acidotisch behandelten Kaninchen 15—25 mg, das maximale 33—57,5 mg, im Mittelwert also 25—36,5 mg. Diese Gewichtszunahme weist, im Verhältnis zu den Kontrolltieren, im Mittelwert 15,8—27 mg, d. h. 171,9—294,5%, auf, was laut statistischer Berechnungen eine signifikante Differenz von 5,27—12,12 bedeutet.

4. Histologisch bestand die Substanz der Nebenschilddrüsen bei den *Kontroll*kaninchen besonders aus *dunklen Hauptzellen*, helle Hauptzellen waren in verhältnismäßig kleiner Zahl vorhanden, an den Randteilen lagerten auch oxyphile Zellen zerstreut. In den vergrößerten Nebenschilddrüsen der *behandelten Kaninchen* hingegen war Blutreichtum festzustellen, die dunklen Hauptzellen nahmen an Zahl erheblich ab, die oxyphilen Zellen fehlten sogar oder nahmen gleichfalls an Zahl ab, die *hellen Hauptzellen* vermehrten und vergrößerten sich aber erheblich, an mehreren Stellen mit Vacuolenbildung, so daß die Hauptmasse der Drüsen von den hellen Zellen gebildet wurde.

5. Auf Grund der Vergrößerung und des histologischen Bildes der Nebenschilddrüsen kann auf ihre gesteigerte Funktion geschlossen werden. Darüber werden wir uns aber nur nach der funktionalen Untersuchung der vergrößerten Nebenschilddrüsen aussprechen können.

6. Unsere früheren Behauptungen, daß bei so behandelten Tieren Hypophysenvergrößerung, Vermehrung der basophilen Zellen des HVL, Vergrößerung und gesteigerte Funktion der Nebennierenrinde, Vergrößerung der Eierstöcke, Follikelreifung und -blutung, Corpus luteum-Bildung, Uterusvergrößerung und Milchsekretion hervorgerufen werden können, weisen darauf hin, daß diese Veränderungen, aber auch die Vergrößerung der Nebenschilddrüsen durch die gesteigerte Funktion des HVL bedingt werden.

7. Die gesteigerte Funktion des HVL ist, unserer Ansicht nach, nicht auf die spezifische Wirkung der verwendeten Verbindungen zurückzuführen, sondern vielmehr auf die von ihnen ausgelöste, periodische acidotische Einwirkung von mäßigem Grade.

8. Unsere Untersuchungen bieten einen neuen Beweis dafür, daß die Funktion des innersekretorischen Drüsensystems durch entsprechende Verabreichung einfacher, acidotisch wirkender Verbindungen umgestellt bzw. gesteigert werden kann. Auf Grund unserer Ergebnisse geht es zu hoffen, daß gegebenenfalls die Funktion der vermindert

funktionierenden Nebenschilddrüsen (und andrer innersekretorischen Drüsen) wiederhergestellt bzw. normal gemacht und zu gleicher Zeit die durch diese unzulängliche Funktion bedingten pathologischen Zustände behoben werden können.

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