GLYCEROPHOSPHATES #110

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MONOGRAPH

ON THE

GLYCEROPHOSPHATES

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GLYCEROPHOSPHATES

Summary

Very little interest has been demonstrated in experimentation with the glycerophosphates; fewer than two dozen papers have been found that warrant review, and more than half have been by foreign authors, requiring translation. Of the five salts comprising the subject of this monograph: calcium, magnesium, manganese, potassium and sodium glycerophosphates, the chemistry of all but magnesium glycerophosphate has been described. The magnesium and manganese glycerophosphates do not appear in the Merck Index, 8th Edition; calcium glycerophosphate is used in dentifrices; baking powder, food stabilizer and as a dietary supplement for calcium and phosphorus (oral dose 0.3 to 1.2 g); the potassium and sodium glycerophosphates are noted as tonics.

All but one of the papers comprising the information sources for this monograph deal with either the sodium or the calcium glycerophosphate; the biological and biochemical sections are not divided on the basis of the separate salts but are considered as a whole. The paucity of information also warrants this treatment.

No toxicity studies were found; most of the papers appear among special studies ranging from anti-teratogenicity to pharmacology. Landauer and Sopher (232) found that the teratogenic effects of various known teratogens may be reduced or alleviated when introduced into fertile chicken eggs accompanied by high-energy substances such as Succinates, glycerophosphates and ascorbate. Sodium glycerophosphate greatly reduced the incidence of muscular hyperplasia, upper beak abnormalities and cervical vertebrae deformation produced by the teratogen, 3-acetylpyridine. Similar studies are discussed by the authors as indicating that teratogenesis is related to interference with mitochondrial functions, and that antiteratogens like glycerophosphate stimulate cellular energy production and lessen the influence of teratogens upon embryonic growth.

A paper by Popoff (315), nearly thirty years earlier than the foregoing, proposed that tumor growth could be controlled by elevating the general cellular functions of the body and endocrines. The theory of Popoff was essentially an early (1942) effort to "cure cancer"; a curious mixture of various salts, glycerine, iodine, vitamins, filtered boiled earth and calcium glycerophosphates, etc. was tested by daily subcutaneous injections of mice with implanted tumors. Popoff reported retardation of tumor growth as long as treatment continued; when treatment ceased, tumor growth renewed. Various feeding experiments produced similar results, but the mortality rate among the treated was rather high.

The relative merits of various dietary calcium supplements were studied by Worten et al. (408) who fed mice calcium carbonate, lactate, di-

or triphosphates or the glycerophosphate. Leg bone samples tested before and after 6 weeks on each of the six calcium diets showed markedly greater bone strength in those animals given the glycerophosphate supplement.

A similar test of the anti-cariogenic properties of calcium glycerophosphate was demonstrated by a Russian experiment conducted by Federov (140) who fed Wistar rats cariogenic diets and brushed their teeth daily with various toothpastes differing principally in containing calcium glycerophosphate, or sodium fluoride or simply the other base materials comprising the bulk of the paste. Serum phosphorus and calcium determinations were made at sacrifice of animals, and the teeth were examined for caries. No significant serum phosphorus or calcium concentration differences were observed or correlated to frequency of caries, but those animals receiving the tooth cleaning with the paste containing calcium glycerophosphate had the largest number free from caries and the minimum number of total caries. Bowen (52) also observed the cariostatic effect of calcium glycerophosphate which he included in the diet of monkeys fed high carbohydrate diets for 22 months; those animals with supplementary glycerophosphate (5 total) showed only 5 caries compared with 47 caries among 6 controls. All the controls had caries, only 2 of the experimentals. Regolati and Hotz (327) added various glycerophosphates or calcium chloride to a cariogenic diet in rats over a period of 3 weeks following 3 weeks on a standard diet. Smooth surface and fissure caries were most numerous on the chloride diet and lowest on phosphates. Calculus deposits, however, were most numerous among the glycerophosphate-fed. There also appeared a distinction between the beta and alpha forms of the glycerophosphates; the beta-glycerophosphates inhibited the formation of smooth surface caries more effectively than the alpha form.

Intravenous sodium beta-glycerophosphate accelerated the healing of fractured bones in rabbits as shown in experiments by Armstrong et al. (13). Artificially fractured radii in 27 test rabbits and 22 controls were compared following treatment of the test group which received 6-14 daily ear-vein injections of 0.1 g sodium beta-glycerophosphate in 4 ml solution; the controls received 4 ml saline. After sacrifice, examination of the fractured radii showed larger fracture calluses and more advanced healing in the glycerophosphate treated than in controls. Doses as large as 1 g glycerophosphate given intravenously to rabbits showed no ill effects. A 17 kg dog given daily intravenous doses of 10 g in 40 ml solution for 10 days showed no observable ill effects or pathologies.

Blood can be prevented from coagulating indefinitely by addition of 2% or greater concentrations of sodium glycerophosphate according to Barnard (22). Both the alpha and beta forms were equally effective as anticoagulants.

Toal and Phillips (377) made a pharmaceutical study of glycerophosphates in 1949 which indicated that the standards of the British Pharmaceutical Codes were not being met by commercial products. A number of changes to be made in BPC monographs for sodium and calcium glycerophosphates were recommended by the authors. A similar Brazilian study had been reported in 1944 by da Costa (103) who represented

glycerophosphates as "highly esteemed and assimilable nerve tonics". The complexity of calcium glycerophosphate and details of its chemical analysis lead the author to suggest changes in the pharmaceutical code.

Velasquez (391) stimulated much interest in the Spanish-speaking countries by his report in 1948 on the pharmacology of sodium glycerophosphate and its use as a uterine spasmolytic and respiratory analeptic. Premature or dangerous miscarriages could be relieved, the contractions of hypertonic dystocias normalized and placental "clampings" overcome by injections of sodium beta-glycerophosphate. Anesthetic intoxication or toxemia of the respiratory centers could be relieved by intravenous 1 to 5 ml of 50% sodium glycerophosphate. It was recommended in clinical obstetrics. Further pharmacological studies carrying on the work of Velasques were reported in some detail by Fernandez and Aguilar (379) a few years later, using rabbits to demonstrate the therapeutic value of sodium beta-glycerophosphate as a modifier of insulin in producing hypoglycemia in the therapy of diabetic coma. Aragon (12) repeated much of the historical information given us by Velasquez and the pharmacology of sodium beta-glycerophosphate as a uterine antispasmatic and respiratory analeptic. There was some theoretical discussion by the author regarding the possible mechanisms underlying these characteristics of the glycerophosphate which render it an effective antispasmotic and respiratory analeptic. Aragon describes some experiments with whole uterus and intestine of guinea pig in which it is shown that the organs, maintained in Ringers solutions, demonstrate the relaxation effect of glycerophosphate added following the creation of uterine spasms by pituitrine, ergotamine or other spasmodic agents. A similar relaxation was not observed in the intestine, further demonstrating the remarkable special tropism of uterine muscle for the glycerophosphate. That is, the action - antispasmodic, of glycerophosphate appears to be specifically upon the uterus alone.

Several papers have been included in the Biochemical portion of the monograph as effects on enzymes and other biochemical parameters. Jacobsohn and Cruz (200) observed that potassium glycerophosphate administered subcutaneously in guinea pigs in doses ranging from 100 to 500 mg resulted in a 15% increase in enzyme activity (glycerophosphatase) 30 minutes after injection of the glycerophosphate.

The influence of glycerine and glycerophosphate upon cholesterol metabolism in experimental atherosclerosis was studied by Liberman (244). Rabbits were given diets containing pure cholesterol, 500 mg/kg daily for about 4 months to produce atherosclerosis. For another 4 months 3 different diets were given to separate groups of animals: continued cholesterol, cholesterol plus glycerine, and cholesterol plus calcium glycerophosphate. The latter additions to the cholesterol were given at a level of 50 mg/kg. Periodic measurements of serum and organ cholesterol levels were made. Cholesterol alone produced increased levels of cholesterol in tissues, organs and blood; the addition of glycerine produced the same effects but at higher levels. Glycerophosphate produced an earlier, more noticeable hypercholesterinemia, and also showed increased aorta levels when compared to cholesterol alone in the diet. Babina and Sidorenkov studied the role of the liver in rendering cholesterol harmless and the effect on this function of glycerine and glycerophosphate. The

addition of glycerine and calcium glycerophosphate to the diet of rabbits increased only the level of esterified cholesterol in the blood and had no effect on the cholesterol level. Tertychnaya (373) tested rabbits in a similar manner on prolonged diets with calcium glycerophosphate without addition of cholesterol. After 5 months on diets containing 200 mg/kg of calcium glycerophosphate the animals were sacrificed, organs removed, and total lipids and cholesterol determined. A slight increase in blood cholesterol was noted but the total serum lipid content was not increased. A significant cholesterol increase was seen in liver, lung and kidney tissues. No atherosclerotic plaques were observed in the blood vessels, but the author did suggest an atherogenic property of glycerophosphate due to the increased blood cholesterol and tissue concentration of lipids and cholesterol.

Prolonged feeding of cholesterol and calcium glycerophosphates inhibited tissue respiration and produced atherosclerosis in rabbits according to a report by Malysheva (254). Daily doses of 0.5 g cholesterol and 50 mg/kg calcium glycerophosphate in the diet for 4 months produced significant lowering of tissue respiration rates in brain, liver and heart muscle tissues. A similar report by Malysheva (253) showed similar results from diets of either glycerine or calcium glycerophsophates.

CALCIUM GLYCEROPHOSPHATE

Chemical Information

I. Nomenclature

A. Common Names

1. Glycerophosphoric acid calcium salt
2. Phosphoric acid glycerol esters calcium salt
3. Calcium glycerophosphate

B. Chemical Names

1. Beta-glycerophosphoric acid calcium salt
2. Alpha-glycerophosphoric acid calcium salt (D,L-forms)
3. Calcium glycerophosphate

C. Trade Name

Neurosin

D. Chemical Abstracts Registry Number

000126-95-4

II. Empirical Formula

$C_3H_7CaO_6P$

III. Structural Formula

$(HOCH_2)_2CHOPO_3Ca$, beta-acid salt
$HOCH_2CH(OH)CH_2OPO_3Ca$, alpha-acid salt

IV. Molecular Weight

210.15

V. Specifications

Food Chemicals Codex:

Assay. Not less than 98.0% $C_3H_7CaO_6P$ after drying
Limits of impurities:

Alkalinity	Passes test
Arsenic (as As)	Not more than 3 ppm (0.0003%)
Heavy metals (as Pb)	Not more than 40 ppm (0.004%)
Lead	Not more than 10 ppm (0.001%)
Loss on drying	Not more than 12%

VI. Description

A. General characteristics

Fine, white, odorless, almost tasteless powder, slightly hygroscopic.

B. Physical properties

Soluble H_2O, one gram per 50 ml at 25 degrees; citric or lactic acids increase solubility as does a lower temperature; almost insoluble boiling H_2O or in alcohol; aqueous solution alkaline.

C. Stability

Store in tight containers.

VII. Analytical Methods

A. Saturated solution gives positive test for calcium (Food Chemical Codex, pg. 926).

B. 100 mg sample heated with 500 mg potassium bisulfate gives pungent vapors of acrolein.

An absorption analysis technique for calcium glycerophosphate is given in a paper by Carl Olof Bjoerling (47):

> *Procedure.* The exchanger used was Amberlite IR-100 H from Nordiska Armaturfabrikerna, Linköping. To avoid swelling during the experiments the resin is kept in water for a day and then poured into a glass tube, 1 cm wide, to a height of 20 cm. When fixed vertically the column permits water to flow through it rather freely without suction. The rate of flow is regulated by a stop-cock at the lower end. The resin is washed with water until the washings are colourless and neutral to methyl red. Then 50 ml of hydrochloric acid 5M is filtered through the column (rate of flow: 5 ml per minute) and the excess of acid removed by washing with 200—500 ml of water (test with silver nitrate or methyl red). The column is then ready for the analysis.
>
> *Blank value.* 100 ml of water run through the column and titrated with 1/10 normal sodium hydroxide (phenolphthalein) consumed 0,15 ml. With "equilibrium water" the value was 0,13 ml. Smaller quantities of water gave proportionally smaller blanks. All figures given below are corrected accordingly.
>
> *Capacity.* The exchanging capacity of the column was tested in the following way. 20 ml of 1/10 molar sodium chloride solution was filtered through the column which was eluted completely with water (25 ml). The hydrochloric acid in the filtrate was titrated with 1/10 normal sodium hydroxide. Indicator: phenolphthalein. The process was repeated with further quantities of sodium chloride. Results see Table 1.

Table 1 Testing the Capacity of the Column

Millival Na Cl introduced	Millival HCl titrated
1,998	1,998
2,998	2,994
4,994	4,930
5,992	5,840

Thus, 2 millival could be used for the column.

Table 5. Calcium Glycero-phosphate

	Per cent			
	Found	Mean	Theory	Yield
Calcium, titration to pH 3,5 ...	16,28; 16,45	16,37	16,28	100,6
Acids, calculated as $C_3H_7O_6P$ ...	69,8; 69,2	69,5	69,08	100,6

5. *Novalgin*, the sodium salt of amidopyrine methane-sulfonic acid (1 H_2O), free from chloride and sulfate. The loss of weight at 105° was 5,3 % (according to formula 5,1 % H_2O).

About 0,4000 g (1 millival) dissolved in 20 ml of water. The usual quantity 2 millivals, gave low yields (2—3 per cent loss). Elution with 50 ml of water. By a potentiometric titration it was ascertained that phenolphthalein was the correct indicator. The approximate content of amidopyrine was determined oxidimetrically with permanganate in alkaline solution by Schulek and Menyhárth's method.[2]

VIII. Occurrence

GRAS as nutrient and dietary supplement (calcium and phosphorus: oral dosage 0.3-1.2 g). Also used in dentrifices, baking powder and as food stabilizer.

MAGNESIUM GLYCEROPHOSPHATE

Chemical Information

I. Nomenclature

A. Common Name

Magnesium glycerophosphate

B. Chemical Name

Magnesium glycerophosphate

C. Trade Name

None

D. Chemical Abstracts Registry Number

000927-20-8

II. Empirical Formula

$C_3H_7MgO_6P$

III. Structural Formula

Not found

IV. Molecular weight

194.39

V. Specifications

No information found

VI. Description

No information found

VII. Analytical Methods

No information found

VIII. Occurrence

No information found

MANGANESE GLYCEROPHOSPHATE

Chemical Information

I. Nomenclature

A. Common Name

Manganese glycerophosphate

B. Chemical Name

Manganese glycerophosphate

C. Trade Name

None

D. Chemical Abstracts Registry Number

001320-46-3
007558-62-5
(Not in Food Additives Dictionary)

II. Empirical Formula

$C_3H_7MnO_6P \cdot xH_2O$

III. Structural Formula

None found

IV. Molecular Weight (anhydrous)

225.00

V. Specifications

Food Chemicals Codex:

Assay	Not less than 98.0% $C_3H_7MnO_6P$ after drying
Loss on drying	Not more than 12%
Limits of impurities:	
Arsenic (as As)	Not more than 3 ppm (0.003%)
Heavy metals (as Pb)	Not more than 40 ppm (0.004%)
Lead	Not more than 10 ppm (0.001%)

VI. Description

A. General characteristics

White or pinkish white powder; odorless and nearly tasteless.

B. Physical properties

Soluble, one gram per 5 ml citric acid solution (1 in 4); slightly soluble H_2O; insoluble in alcohol.

C. Stability

Store in well-closed containers.

VII. Analytical Methods

A. A 1 in 20 solution in dilute HCl test solution gives positive test for manganese (Food Chemical Codex, pg. 927).

B. 100 mg sample heated with 500 mg potassium bisulfate gives pungent vapors of acrolein.

VIII. Occurrence

GRAS as nutrient and dietary supplement.

POTASSIUM GLYCEROPHOSPHATE

Chemical Information

I. Nomenclature

A. Common Name

Potassium glycerophosphate

B. Chemical Names

Potassium glycerophosphate
Potassium glycerophosphate, trihydrate
Potassium glycerophosphate, anhydrous

C. Trade Name

None

D. Chemical Abstracts Registry Number

0001319-70-6, Potassium glycerophosphate, and the trihydrate
0001319-69-3, Potassium glycerophosphate, anhydrous

II. Empirical Formula

$K_2C_3H_7PO_6 \cdot 3H_2O$ (trihydrate)

III. Structural Formula

Not found

IV. Molecular Weight

302.31

V. Specifications

Food Chemicals Codex:

Assay	$C_3H_7K_2O_6P \cdot 3H_2O$, not less than 80.0% $C_3H_7K_2O_6P$; <u>potassium glycerophosphate solutions</u>, not less than 95.0% and not more than 105.0% labeled concentration of $C_3H_7K_2O_6P$.
Limits of impurities:	
Arsenic (as As)	Not more than 3 ppm (0.003%)
Heavy metals (as Pb)	Not more than 20 ppm (0.002%)
Lead	Not more than 5 ppm (0.0005%)

VI. Description

A. General characteristics

Pale yellow, syrupy liquid or viscous mass; with 3 molecules water of hydration; usually marketed as colorless or pale yellow syrupy solution (50-75% aqueous).

B. Physical properties

Very soluble H_2O; alkaline to phenolphthalein or litmus.

C. Stability

Store in tight containers.

VII. Analytical Methods

A. A 1 in 10 solution gives positive test for potassium (Food Chemical Codex, pg. 928).

B. 100 mg sample heated with 500 mg potassium bisulfate gives pungent vapors of acrolein.

VIII. Occurrence

GRAS as nutrient and dietary supplement. Has been used as a tonic.

SODIUM GLYCEROPHOSPHATE

Chemical Information

I. Nomenclature

A. Common Name

Sodium glycerophosphate

B. Chemical Name

Sodium glycerophosphate

C. Trade Name

None

D. Chemical Abstracts Registry Number

000819-83-0 (not in Food Additives Dictionary)

II. Empirical Formula

$C_3H_7Na_2O_6P$

III. Structural Formula

$Na_2C_3H_5(OH)_2PO_4 \cdot 5\frac{1}{2}H_2O$, beta-form
$(HOCH_2)_2CH \cdot O \cdot PO(ONa)_2 \cdot 5H_2O$ (Lange's Handbook of Chemistry)

IV. Molecular Weight

315.15
306.14 (Lange's Handbook of Chemistry)

V. Specifications

None found

VI. Description

A. General characteristics

White, odorless, scale-like crystals (beta-form); alpha-form syrupy.

B. Physical properties

Decomposes above 130 degrees; soluble in about 1.5 parts water; more soluble in hot water; insoluble in alcohol; aqueous solution alkaline, pH about 9.5.

C. Stability

Store in tight containers.

VII. Analytical Methods

None found

VIII. Occurrence

Has been used as a tonic.

GLYCEROPHOSPHATES

Biological Data

I. Acute Toxicity

No information found

II. Short-Term Studies

No information found

III. Long-Term Studies

No information found

IV. Special Studies

Teratogenicity (Anti)

The teratogenic effects of certain substances introduced into fertile chicken eggs may be reduced or alleviated when the teratogen is accompanied by high-energy intermediates such as succinates, glycerophosphates and ascorbate fed into the mitochondrial respiratory chain. Landauer and Sopher (232) experimented with various teratogen-antiteratogen combinations using fertile chicken (White Leghorn) eggs which had been incubated for 96 hours and were then injected with the test substances into the yolk sac. After 18 days of further incubation, the embryos were removed and examined for gross abnormalities. Only the studies with sodium glycerophosphate will be described here.

Two tests of possible interactions of 3-acetylpyridine and glycerophosphate were conducted; the toxicity of the pyridine was greatly reduced when accompanied by glycerophosphate. The incidence of muscular hypoplasia, upper beak abnormalities and cervical vetebrae deformation was considerably reduced. The results are shown in the following table:

Table 4. *The effect of treatment at 96 h of incubation with 0·6 mg/egg (4·95 μM) 3-acetylpyridine either alone or in combination with 10 mg/egg (30·86 μM)* DL-*α-glycerophosphate hexahydrate disodium salt*

(Supplement and teratogen injected together in the same vehicle.)

	3-Acetylpyridine	3-Acetylpyridine supplemented with glycerophosphate
Treated ...	236	229
Mortality to 18 days (%)	44·5	19·7
Survivors	133	183
Normal appearance (%)	15·8	80·3
Muscular hypoplasia (%)	82·0	19·1
Short upper beak (%)	13·5	0·5
Crooked neck (%)	8·3	0

Similar tests with 6-aminonicotinamide led to an increased embryo mortality, but among survivors, the incidence of micromedia and beak abnormalities was much reduced. Syndactylism occurred more frequently when the glycerophosphate was present, but not significantly so. The results are shown in the following table:

Table 5. *The effect of treatment at 96 h of incubation with 5 γ/egg (0·035 μM) 6-aminonicotinamide either alone or in combination with 10 or 15 mg/egg (30·86 or 46·29 μM)* DL-*α-glycerophosphate hexahydrate disodium salt*

(Supplement and teratogen injected in the same vehicle.)

	6-Aminonicotinamide	6-Aminonicotinamide supplemented with glycerophosphate
Treated ...	251	235
Mortality to 18 days (%)	9·6	19·6
Survivors	227	189
Normal appearance (%)	11·9	41·8
Micromelia (%)	88·1	58·2
Abnormal beak (%)	27·8	14·8
Syndactylism	4·8	6·9
Miscellaneous defects (%)	1·3	0·5

of grossly normal appearance) in the two groups. Syndactylism occurred with slightly higher incidence in the presence of glycerophosphate, but the frequencies were low and the differences not statistically significant.

Our data demonstrate clearly that in the presence of glycerophosphate the teratogenic effects of the two niacin antagonists 3-acetylpyridine and 6-aminonicotinamide are greatly reduced. In contrast, no such beneficial results were

The authors discuss the role of the anti-teratogens when given exogenously to relieve the teratogenic effects of various substances. Compounds previously studied in the manner described in this paper, and shown to be anti-teratogenic, are listed with references. The author summarized published data and conclude that evidence indicates that teratogenesis is chiefly caused by interference with mitochondrial functions and growth of the organism, and that the anti-teratogens serve as a stimulus for cellular energy production and increase the "changes for protection of the embryo against teratogen-induced damage or of its repair".

Carcinogenicity (Anti)

A theory was advanced in 1942 by Popoff (315) for controlling the growth of tumors "by elevating the general cell functions of the body and its endocrine glands with stimulants to normalize the chemical conversions and the growth correlations of the body, and in this way to hem the growth and increase of the tumor cells". The author had experimentally determined that $MgCl_2$, $MgSO_4$, MgBr, KBr, glycerine, $MnSO_4$, KI and $NaSO_4$ used topically on cancer lesions, and subcutaneously, inhibited tumor growth but did not suspend growth completely. The experiment reported in this paper was similarly only partially successful, and utilized a stimulant composed of $MgCl_2$, $MnSO_4$, glycerine, iodine, lithium salts, iohimbine, vitamins, pepper extract, filtered boiled earth (for humus acids), and calcium glycerophosphate!

Groups of 15-20 white mice were implanted subcutaneously with pieces of the same tumor. Half the animals remained untreated as controls. The daily injections of the stimulant solution (half a ml) were given subcutaneously on the side of the body away from the tumor, for several days. Daily suspensions of treatment alternated with injections until 8-10 injections were given. About 300 mice were treated in all and the differences between treated and controls were often striking; the tumors of the treated mice were 1/3 to 1/5 the size of those in controls. Inhibition of tumor growth continued as long as treatments were given; when treatment was suspended, tumor growth renewed. Feeding experiments with lithium and manganese salts added to the milk portion of the diet, with an additional drop of iodine tincture added every third day. This diet produced tumor growth inhibition similar to that of the injected treatments, but the treated animals showed a high rate of mortality among the treated mice. The author suggested that "it is possible that organic compounds will become significant in this area", referring to the tumor growth inhibition shown in the experiments.

Diet Supplement

Worten et al. (408) performed a study of the comparative efficiency of calcium glycerophosphate as a dietary calcium supplement relative to several commonly used calcium compounds such as the carbonate, phosphates and lactate. In the calcification of tissues in bone development, phosphorus is needed as well as calcium. In vitro studies indicated that calcium glycerophosphate, with its minimal antiacid properties and wide range of solubilities, should be an ideal supplement for calcium deficient patients. To supplement the in vitro study the following animal study was done.

Newly weaned female mice were placed in six groups; one group served as controls; the five experimental groups received one of five different calcium sources - carbonate, lactate, di- or tri-calcium phosphate, or the glycerophosphate. The calcium was added to the basic diet as 0.5% concentrations. Leg bone samples were tested before and after 6 weeks of calcium supplementation. The increased strength of the bones of the glycerophosphate-fed group was markedly greater than that of either control or of those fed other calcium salts. The glycerophosphate diet produced the healthiest animals in appearance; the carbonate diet resulted in nervous and excited animals which lost hair and became scabbed. The lactate and phosphate diets resulted in somewhat healthier animals in appearance, but some hair loss occurred and the sleek appearance of the glycerophosphate diet was not observed.

Cariogenicity (Anti)

A Russian experiment in the anti cariogenicity property of calcium glycerophosphate administered topically to the teeth of Wistar white rats given cariogenic diets was described by Federov (140). Four groups of animals 30 days old were placed on a carbohydrate cariogenic diet for 30 days during which various toothpastes were applied daily to the teeth of three of the groups, the fourth group serving as controls. The composition of the toothpastes was as follows: group I had teeth cleaned with a paste consisting of 2.5% calcium glycerophosphate, 2% carboxyl-methyl-cellulose, 29.6% chalk, 43% water, 20% glycerine, and 2.9% NaCl; group II, paste consisted of 0.1% sodium fluoride, 2% carboxyl-methyl-cellulose, 44.2% chalk, 26.9% glycerine, and 26.9% water; group III, paste composed of 1.2% carboxyl-methyl-cellulose, 43.4% chalk, 27.1% water, and 27.7% glycerine; group IV rats did not receive the tooth-cleaning treatment.

At the end of the experiment, the animals were sacrificed, blood phosphorus and calcium levels were determined, and the jaws were cleaned and examined microscopically for caries. Of the 11 animals in group I, 3 were free of caries, and the minimum number of caries in all occurred in this group. The sodium fluoride paste of group II, and the general paste of group III were less effective in preventing or reducing caries; the maximum caries were found in group IV. No significant differences were observed in serum phosphorus and calcium concentrations in the experimental groups, nor was the frequency of caries sex related. Calcium glycerophosphate was the most active preventive of caries in the white rat.

The cariostatic effect of calcium glycerophosphate was presented in a paper by Bowen (52) who described experiments with monkeys, 5 experimental and 6 controls, fed high carbohydrate diets (including icing sugar 5 times daily) over a period of 22 months. The experimental animals received 1% calcium glycerophosphate in the diet. Controls showed a total of 47 carious lesions; experimentals, only 5. All controls developed dental caries; only 2 experimentals showed caries, and the total phosphate in plaques was higher than that in controls.

Regolati and Hotz (327) performed experiments with rats to determine the cariostatic effects of various glycerophosphates added to a cariogenic diet at 0.6% phosphorus level. Ten liters of Osborne-Mendel rats, 9 animals each were given standard finely powdered diets for 3 weeks then the test groups were given various phosphorus-supplemented diets for 3 weeks after which the rats were sacrificed. The jaws were removed and plaques evaluated microscopically; smooth surface lesions and carious lesions were scored. The eating patterns of the animals showed small but significant differences; controls ate less frequently than the test groups, the test animals on calcium chloride ate longer and those on calcium glycerophosphate ate less than those on any other diet. The incidence of smooth surface and fissure caries was highest in the chloride diet and lowest in the phosphates, but the calculus deposits were most numerous in the glycerophosphate diet. There was a distinction between the beta and alpha forms of the glycerophosphates in that the beta form was a more effective inhibitor of smooth surface caries than was the alpha glycerophosphate.

Therapy

The effect of intravenously injected sodium glycerophosphate (the beta form) on the healing of fresh bone fractures in rabbits was studied by Armstrong et al. (13), who considered that a low plasma bone phosphatase might be responsible for a delayed calcification in repair of fractures. Twenty-seven rabbits in the test group, and 22 animals as controls had the right radius fractured by forceps; the experimental group received 6-14 daily injections by ear vein of 0.1 g sodium beta-glycerophosphate in 4 ml solution and the controls received 4 ml saline. After the last injection, the rabbits were sacrificed and the fractures studied. The animals treated with the glycerophosphate showed larger fracture calluses and more advanced healing. Analyses of serum inorganic phosphate and calcium made at frequent intervals showed no consistent differences between treated and control animals. Rabbits given doses of the glycerophosphate as large as 1 g intravenously daily showed no ill effects; 26 daily subcutaneous doses of 0.1 g in rabbits produced no pathological findings at autopsy. A dog weighing 17 kg was given daily intravenous injections of 10 g in 40 ml solution for 10 days without observable ill effects or pathologies. Three to six grams of glycerophosphates daily were fatal in rabbits intraperitoneally injected for 2-5 days. The authors suggest that glycerophosphates in a wide range of doses appear to be innoxious and should be safe for human application in cases where fractures heal more slowly than usual.

Coagulation (Anti)

Concentrations of sodium glycerophosphate of 2% or greater will prevent blood coagulation indefinitely in most instances, reported Barnard (22) in studies with freshly drawn human blood. Lower concentrations of the glycerophosphate delay coagulation; a 1% solution is effective for several hours and concentrations as low as 0.04% produce some measurable delay. Clots in glycerophosphated blood appear to shrink more rapidly and become smaller than in untreated blood. Both alpha and beta sodium glycerophosphates were effective as anticoagulants.

Pharmacology

Toal and Phillips (377) presented the results of a pharmaceutical study in 1949 to draw attention to an unsatisfactory position of the glycerophosphates standards in the British Pharmaceutical Codex. Commercial glycerophosphates were not satisfying the full requirements of the Codex. The preparation and assay of sodium isomers, alpha and beta salts were described, as were the calcium salts. Analyses of commercial preparations of calcium glycerophosphate showed 3 of 4 samples failing to meet the Codex standards. A number of changes to be made in the BPC monographs for sodium and calcium glycerophosphates were recommended by the authors.

A Brazilian pharmacological study of calcium glycerophosphate was presented in 1944 by da Costa (103). Glycerophosphates are "highly esteemed and assimilable nerve tonics", and are "recommended in all cases where we need to raise the level of organic phosphorus. Three categories of glycerophosphoric esters are described: the glycerophosphoric monoesters; glycero-diphosphoric diesters; and glycero-monophosphoric diesters. The complexity of calcium glycerophosphate, a mixture of mono and diesters in variable proportions, was demonstrated by the author in detailed chemical analyses leading to suggested changes in the pharmaceutical code governing the therapeutic use of calcium glycerophosphate.

The pharmacology of sodium glycerophosphate is discussed in a report by Velasquez (391) who describes the use of the pharmaceutical as a uterine spasmolytic and respiratory analeptic. Unlike the general spasmolytic action of glycerine on all smooth muscle, the sodium glycerophosphate affects the uterine muscle only and has proven to be a useful medication in numerous obstetrical events. Premature or dangerous miscarriages can be relieved; injections of sodium beta-glycerophosphate can normalize the contractions of hypertonic dystocias; and placental "clampings" may be overcome, freeing the placenta for normal discharge.

Toxemia of the respiratory centers, or anesthetic intoxication, can be relieved by intravenous injections of 1 to 5 ml of 50% solution of sodium glycerophosphate which greatly stimulates respiration. When administered suboccipitally, the effect is most pronounced. Velasquez recommends the use of sodium beta-glycerophosphates in clinical obstetrics.

Reference is made to the pharmacological studies of Velasquez and his school by Fernandez and Aguilar, (379) who discuss experiments with rabbits demonstrating the therapeutic value of sodium beta-glycerophosphate as a modifier of the action of insulin.

Fasted adult rabbits, weighing between 1.75 and 2.5 kg, selected without regard to sex, were treated in a series of experiments as follows: 2 rabbits (1.8 and 2.5 kg) were injected subcutaneously with 500 mg/kg Na beta-glycerophosphate; 1 animal (2.35 kg) was first injected sub-

cutaneously with 2 units insulin and later with 2 units insulin plus 500 mg Na-beta glycerophosphate/kg; 1 animal (2.15 kg) received in the same manner 2 units insulin plus 750 mg/kg Na beta-glycerophosphate plus 500 mg glucose 150 minutes after the insulin-glycerophosphate injections; an additional rabbit (1.75 kg) was similarly treated. Two more experiments completed the series: 1 rabbit received (2.25 kg) the 2 units/kg insulin, and the last animal (2 kg) received 2 units insulin plus 1.25 mg ATP/kg body weight.

After each treatment blood samples were taken at 30 minute intervals and blood glucose determined by Folin-Malmros micromethod; this method is described by the authors. The role of the glycerophosphate in the metabolism of carbohydrates is described as similar to that of ATP, both producing hypoglycemic reactions, reinforcing the action of insulin in the therapy of diabetic coma. The authors note corroborative works showing the beneficial use of sodium beta-glycerophosphate in the therapy of diabetic coma.

Spanish and South American experiences with the use of sodium beta-glycerophosphate as a uterine anti-spasmodic agent and as an analeptic was outlined in a report by Aragon, (12). The pharmacology of the substance is discussed in some detail with respect to sodium beta-glycerophosphate action as a uterine anti-spasmodic and as a respiratory analeptic. The author delineates the requirements for a good anti-spasmodic agent and indicates that all requirements are met by sodium glycerophosphate which he considers to be the best of the uterine anti-spasmodic agents known at that time (12). There appears to exist a special tropsism by the smooth fiber of the uterus for the glycero-phosphoric molecule in its sodium salts (an indifferent cation). The author further hypothesizes that the fixation of glycerophosphoric molecule through the phosphate radical then permits sedation by the primary dialcoholic function resulting in relaxation of spasm, a general function of alcohols.

The theoretical discussion of the action of glycerophosphates and the possible mechanisms by which it serves as an analeptic agent on the respiratory centers vaguely associates the glycerophosphates with the acetylcholines, phosphocreatines and ATP; the author suggests that the mechanism will "probably be very difficult to demonstrate".

The author then proceeds to describe some of his experimental studies which demonstrate the antispasmodic action of sodium beta-glycerophosphate, using the extracted whole uterus of the guinea pig, and a repeat-experiment with isolated guinea pig intestine. The test equipment with its Ringer solution for the experimental environment is described. Various solutions producing spasms in uterine and intestinal materials are added: pituitrine, ergotamine, barium chloride and histamine. In each instance, the addition of the spasmodic agent is rapidly counteracted by addition of the glycerophosphate in the uterine preparation; no relaxation is observed in the intestine contraction under similar conditions.

In summary, sodium beta-glycerophosphate has been successfully used in incipient abortion, premature partuition and surgical trauma, and as a respiratory analeptic or relief in newborn infants.

GLYCEROPHOSPHATES

Biochemical Aspects

I. Breakdown

No information found

II. Absorption-Distribution

No information found

III. Metabolism and Excretion

No information found

IV. Effects on Enzymes and Other Biochemical Parameters

Potassium glycerophosphate in doses of 0.1 or 0.5 g was injected subcutaneously into guinea pigs by Jacobsohn and Cruz (200) in an experiment to determine the influence of substrate introduction upon the enzyme phosphatase. The phosphatase level in the blood was determined before injection and at 30 minutes after injection of the glycerophosphate. Enzyme activity (glycerophosphatase) was observed to increase by about 15% at 30 minutes after injection of the glycerophosphate.

Liberman (244) performed experiments with rabbits in studies of the mechanism by which glycerine and glycerophosphate influence cholesterol metabolism in experimental atheroschlerosis. Rabbits of undetermined strain, weighing 2.5-3.0 kg, were fed diets containing pure cholesterol in doses of 0.5 g/kg daily for 4-4.5 months to produce atheroschlerosis. During the next period of similar duration, three different diets were given: continued cholesterol; cholesterol plus glycerine (50 mg/kg); and cholesterol plus calcium glycerophosphate (50 mg/kg). Periodic measurements of serum and organ cholesterol levels were made. The cholesterol diet produced increased levels of cholesterol in tissues, organs and blood, due apparently chiefly to increased esters of cholesterol. The addition of glycerine produced similar effects but at higher levels; glycerophosphate produced an earlier hypercholesterinemia that was most noticeable. The glycerophosphate-cholesterol diet action differs from the cholesterol alone chiefly in the increased aorta levels of cholesterol; in other tissues, cholesterol levels are relatively unchanged from those observed in cholesterol alone.

A similar study, also using rabbits, and the effects of glycerine and calcium glycerophosphate on hypercholesterinemia, was described in

1964 by Babina and Sidorenkov (17). Three groups of rabbits were used: group 1 (control of 29 animals received daily doses of 0.5 g/kg cholesterol in the diet; group 2 (9 rabbits) were given 50 mg/kg glycerine in the diet instead of cholesterol; and group 3 (11 rabbits) received 0.1 g/kg calcium glycerophosphate. The experimental diets lasted for one week. Blood samples taken before and periodically during experimental feeding were analysed for total, esterified and free cholesterol. Hypercholesterinemia which showed in those animals receiving supplementary cholesterol (group 1) was apparently chiefly due to increased esterified cholesterol. The supplementary diets of glycerine and glycerophosphate increased the level of serum esterified cholesterol, but had no effect on free cholesterol in the blood.

The influence of calcium glycerophosphate in the diet upon the levels of cholesterol and lipids in serum and tissues was studied by Tertychnaya (373) using rabbits in prolonged feeding over a period of 5 months. Other investigators had observed that such treatment led to indications of experimental atherosclerosis in the animals, presumably due to the slowness with which cholesterol is oxidized resulting in its deposition in tissues.

Rabbits weighing 2.5-3 kg were given diets containing 200 mg/kg calcium glycerophosphate for 5 months; controls received the same diet without the glycerophosphate. Ten animals comprised each group. The rabbits were sacrificed and organs removed for determination of total lipids and cholesterol; the lipoprotein serum content was also determined. Blood cholesterol was slightly increased, with a corresponding decrease in protein-cholesterol complexes. The total lipid content of the serum was not significantly increased.

The total lipids in heart and skeletal muscle were not increased significantly; a tendency toward an increase was seen, however, in liver, lung and kidney tissues. The cholesterol content significantly increased in the latter tissues. The author concluded that even though atherosclerotic plaques were not observed in the blood vessels, glycerophosphate does possess an atherogenic effect, expressed by the increased blood cholesterol and tissue deposition of lipids and cholesterol.

Inhibition of tissue respiration and development of atherosclerosis in rabbits by prolonged feeding of cholesterol and calcium glycerophosphate was reported by Malysheva (254). Adult rabbits, of about the same age and weight, were given daily doses of 0.5 g cholesterol and 50 mg/kg calcium glycerophosphate in the diet for a period of 4 months. Autopsy after sacrifice showed aorta and other tissues clearly atherosclerotic. Various tissue respiration rates were measured and revealed a statistically significant lowering of rates in brain, liver and heart muscle tissues, presumably due to the action of the prolonged administration of the calcium glycerophosphate in the diet.

The influence of glycerine and of calcium glycerophosphate, given

in the diet of rabbits, on tissue respiration rates was reported by Malysheva (253). Daily doses of 50 milligrams/kg of either glycerine or glycerophosphate in the diet over a period of 5 months reduced the respiration rate in brain, liver and heart muscle tissues significantly. Tissue samples from the sacrificed animals were made into a paste; the respiration rate was determined manometrically in a Warburg apparatus. The glycerine reduction in respiration rates for brain, liver and heart muscle were observed to be 16.7, 39.8 and 53.5% respectively; corresponding values for calcium glycerophosphate were 17.2, 21 and 32%.

VI. Consumer Exposure

No information found

GLYCEROPHOSPHATES

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Experimental Pharmacological Study of the Anti-Spasmodic Action of Sodium-Beta-Glycerophosphate on the Uterus

By

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Professor B. Lorenzo Velazquez initiated the pharmacological study of sodium-beta-glycerophosphate in the grid of the year 1944, demonstrating that it is a uterine anti-spasmodic agent and analeptic agent, as it was later proved by Perez Carnicero and Gareia del Jalon in 1946, in the Madrid Professorship of Pharmacology.

This anti-spasmodic action on the uterus, having been demonstrated at first experimentally, was proved obstetrically by Botella Llusia.

Since then this medication has drawn the attention of some Spanish obstetricians and pharmacologists, who have confirmed its specific uterine anti-spasmodic action.

In South America the subject has been studied by Daniel Carril in Chile and E. Montragail and Oliveiro Pinto in Brazil, from the tocologic point of view.

In Peru, it has not even yet been tried, with Professor Doctor Carlos A. Bambaren, being the only one who is studying it in the pharmacology Professorship of the Pharmacy Faculty in Lima.

Sodium Glycerophosphate and Its Pharmacological Properties

The study of the pharmacologic properties of sodium glycerophosphate begins with the works of B. Lorenzo Velazquez and his collaborators Garcia del Jalon and Perez Carnicero, in Spain, in 1944, as a consequence of investigating the action of glycerine on smooth musculature.

These authors tried to explain the coming action that the intravenous injection glycerine produces in hepatic and nephoitic spasms, and it confirmed that glycerine is in vitro an anti-spasmodic agent for all the organs and smooth musculature. This affect is attributed to the polyalcoholic function, since even the fatty monoalcoholic function to bodies of certain molecular weight and of certain chemical structure possesses relaxing properties on the contracted smooth fiber, as Perez Cirera demonstrated in 1938 for octylic alcohol, and later Garia del Jalon (1944) demonstrated for propylic phenyl, while B. Lorenzo Velasquez and Perez Carnicero were those who demonstrated the anti-spasmodic action of the polyalcohols (21).

Of the alcohols tried in pharmacology the least toxic is glycerine, but its use turned out to be a bit dangerous for the hemolisis that it produces. That led Lorenzo Velazquez and collaborators to study other substances, which carrying glycerine in their molecules might have the actions of this trial propane (sic) without toxic effects. They studied the glycerophosphates, managing to discover that sodium glycerophosphate markedly reduces the contractions of the uterine muscle in spasm, an action which it does not exert on the other organs of smooth musculature.

Of the two isomacros, the Beta derivative turned out to have a much more pronounced action than the Alpha derivative (20).

Of the glycerophosphates tried by the above mentioned authors, only sodium and magnesium behave as anti-spasmodic agents, while calcium, zinc, iron and magnesium increase the spasms and contractions; in the latter the respective cation dominates over the glycerophosphatic group.

Magnesium glycerophosphate in equal amount with sodium glycerophosphate turns out to be more active (because the intense anti-spasmodic action produced by the magnesium ion is added to the action of the glycerophosphoric radial) but its action is not specific for the uterine fiber, and moreover, it suppresses the natural movement of the organ as well as the spasms, and furthermore it brings with it the toxic action of the magnesium ion. (20) (22).

The anti-spasmodic action of sodium-beta-glycerophosphate was studied later, in 1947, by L. Cuny and D. Quivy (5), in France, comparing it with the action of glycerine and sodium-alpha-glycerophosphate, while reaching the same conclusions as those of Professor Lorenzo Velazquez.

In the test carried out in the obstretical clinic, in connection with its paralyzing activity on the uterus, the investigators observed that the infants whose mothers were treated with sodium glycerophosphate displayed a considerable decrease in respiration.

This fact caused an interest in studying experimentally its action on the respiratory centers. Perez Carnicero, Garcia del Jalon, and Urtasum (12) found that the intravenous administration of this salt produced a considerable increase in the respiratory range in the respiratory centers of animals intoxicated with carbon dioxide. This increase in range and frequency is more intense when the injection is performed suboceipitally, acting in this case on arterial pressure that it decreases.

It is to be noted here that, because of being related to the action mechanism of sodium glycerophosphate, glycerine in the same conditions and concentrations, apart from its hypotensor action, produces no effect.

In 1946 Lorenzo Velazquez, Felix Saenz Sanchez and F. Javier de Ello (17) while attempting to investigate the action mechanism of sodium glycerophosphate, studied the modifications in the phosphatic activity of blood serum by the intravenous administration of sodium beta glycerophosphate in dogs, arriving at the following conclusions:

1. Sodium beta glycerophosphate injected endovenously into the dog, produces a marked decrease in the phosphatic activity of the blood serum.

2. The recovery of this activity is attained between 1-24 hours after the administration.

From the foregoing, it may be concluded that the principle pharmacological actions are:

b). Anti-spasmodic action on the uterus.
a). Respiratory analeptic action.

The Anti-Spasmodic Action on the Uterus of Sodium Beta Glycerophosphate

The uterus is one of the organs of smooth muscularture, whose motivity is controlled, on the one hand, by the influence of the vegatative nervous system, and of the other, it possesses within itself the cause of its own motility. (16).

Physiologically, the uterus, the same as the other organs of smooth musculature, is intensely inhibited and it is only modified action with its physiological function.

The use of substances which modify uterine contractibility produces various results according to the period in which they are administered (9) (18).

This organ displays hypotoxicity or hypertoxicity, resorting in these cases to oxitocins or anti-spasmodic agents (18).

The substances which act upon the uterus, increasing its motility, are the oxitocins, while those that decrease the tone of the uterus, reducing the frequency of its contractions, and reducing its size, are the anti-spasmodic agents (9). In this last group of substances sodium beta glycerophosphate is considered important because of the excellent actions that it possesses demonstrated both in the clinic and in pharmacological experimentations.

Any good antispasmodic agent must include the following traits: (12)

1. Produce a rapid and energetic sedation of the uterus.

2. That it does not inhibit the rhythmic or spontaneous contractions, nor prevent the action of the uterus to antispasmodic drugs.

3. That it does not cause colateral nor undesirable affects on other organs or systems.

4. It must be harmless to the mother and child, in addition to responding by its integrity to the physiological limber of its partition. (18).

The filtrate is the most difficult to find, in as much as most medicines that relax the smooth fiber of the uterus, behave analogically on the muscular system of the intestine, of the muscles, or even provoke other toxic actions (3) (9) (13).

Sodium glycerophosphate includes in an excellent fashion these qualities, in particular the third which is so difficult to find in antispasmodic medicines. Because of these properties, it deserves to be considered as the best of the uterine antispasmodic agents known up to this time, with the exception of lutein which, because of being less foreign to the organism, rather for physiological is better tolerated by it.

Sodium beta glycerophosphate constitutes a uterine antispasmodic agent

of purely symptomatic action, it acts by suppressing the spasms; although still new, nevertheless, the clinical tests performed are favorable to its use.

The use of sodium beta glycerophosphate in obstretics. Sodium beta glycerophosphate because of its great role as an antispasmodic agent in the uterus has found multiple indications of obstretics. Botella Lusia and Garcia Funcasta were the first to try it and to establish the bases for it use, of the amounts of which it should be used in each case.

It was used in:

1. Incipient abortion.

2. Prevention of abortion and premature partuition, after surgical traumatism.

3. Treatment of premature partuition.

In these cases sodium beta glycerophosphate constitutes a symptomatic medicine, but its effectiveness surpasses that of citogenic treatments, (Lutein, Vit. E) (1) (2) (4) (10) (14).

Several followers have been observed, which the authors have attributed to different causes, such as an incorrect indication, or insufficient amounts, while taking special note of the fact that the medication failed when it was a question of women with neuroitic case histories. Botella Llusia and Garcia Funcasta and Roncales Cativiela (2) (14), propose the association of the treatment with vitamin B_1 and in some cases with progestinone [sic] [progesterone?] and tekoferol.

Action mechanism of sodium beta glycerophosphate. The action mechanism of sodium beta glycerophosphate is subject to mirror work hypotheses.

The uterotropal action of this medicine is outstanding, in such a way that raw glycerine behaves as a antispasmodic agent on all the smooth fiber muscles, sodium beta glycerophosphate relaxes only with regard to the spasms of the uterine organ.

This specific action according to Lorenzo V lazquez (19)seems to be related to the fixation of the glycerophosphoric compound in the smooth fiber of the uterus, in close relationship with the special phosphorated metabolism of the uterine fiber, especially in the present state. The uterus under these conditions displays a special tropism for the glycerophosphoric molecule in its sodium salt (indifferent cation).

This tropism of pure metabolic match is made through the phosphoric radical and acting on adenosol phosphate with the action of Colinesterasa necessary for the genesis of acctilcoline. The fact that the same sodium phosphate reinforces the muscular force of the uterus supports this supposition. (Embden Deuticke) (1941). Consequently sodium beta glycerophosphate would increase the muscular tur contractions but; the fact is that immediately the specific fixation on the uterus of the glycerophosphoric molecule through the phosphate radical, then the primary dialcoholic function produces sedation or relaxation of spasm, the general functions of alcohols. For all that and especially for the fixation of the phosphatic group a certain level of aneurine pirophosphate is necessary, which explains why the medication fails with B^1 avitaminosis.

Now something in the uterus and not in the other smooth muscles which explains the tropism and the specific relaxation of spasms in the uterus by this medicine, be it because of the special phosphoric metabolism for acetilcolinogenesis, more active in the pregnant uterus, or in connection with the Meyerhoff cycle of the hydrocarbonate metabolism, in which glycerophosphate appears as an intermediary, or be it finally, because of a special enzymatic action in the uterus, which specifically separates sodium beta glycerophosphate, leading the polyalcohol free with the consequent relaxation of spasm.

With regard to the action mechanism on the respiratory centers, the difference in action compared with glycerine, whether on this respiration or on blood pressure, permits the finding of a starting point for establishing the possible action mechanism in the favorable action of the glycerophosphoric molecule.

If the injection of glycerine and the injection of sodium glycerophosphate are separately inactive, and the total glycerophosphoric molecule results from marked pharmacological action, it is possible that in double mechanism intervenes in this action. The dialcoholic function remaining freefrom the glycerine residue, was not explained the stimulusbut if the fixation of the glycerophosphate in the nervous centers (respiratory and vasomotores) once that this molecule is fixed the phosphoric radical activates acetilcolinogenesis, in this way remarked stimulus on such centers on specific form on the respiratory ones might be explained.

Since the studies of Nachmanson and collaborators (1941-1943) it is known that colinesterones needs, for its action in the forming aceticoline the presence of adenosine triphosphate, in the recovery of which the division of phosphocreatine happens. The phosphorado or phosphataded radical of the glycerophosphate favors this metabolic cycle in acetilcolinogenesis once that the glycerophosphoric molecule is fixed in the centers by the two free alcohols of the glycero radical. This is within the realm of possibility and it will probably be very difficult to demonstrate it in as much as the metabolism of acetilcolingenesis is not completely done in all its detail.

Investigations performed. I have studied experimentally the antispasmodic action of sodium beta glycerophosphate on the uterus, on the isolated organ of the guinea pig, and the effect that it produces on the isolated intestine.

Technique. The antispasmodic action of sodium beta glycerophosphate may be proved:

1. By decrease in or suppression of the response of the organ to drugs which increase its activity by previous addition of glycerophosphate to the nutritous liquid.

2. By the effect that its addition produces on the organ which was previously in spasm.

I have used this latter method because of considering it more appropriate for appreciating the modifications in uterine activity and because the previous conduction of the spasmodic state which is manifested by the increase in the contractions in the organ, serves as a proof of organ sensitivity to spasmodic substances, necessary in this case for proving the relaxing action of this medicine.

Arrangement. It is set up according to the following system:

Bano Marfa. It is a metallic recipient of several liters capacity with thermoelectric regulations designed to maintain a constant temperature.

Quest container. It is a glass cylinder which at its lower end ends in a thin tube. By means of a rubber tube it is connected with a glass coil closed in the interior of the bath.

The supplying and draining of the test container with Ringer solution is carried out through this coil and by means of a two way cup. Through its large other part, receives the drug to be tested.

Oxygen bubbler. It is formed by a glass tube bent in a U shape, one of the ends of which is connected with a oxygen generator, with the use of all possible (In my experiments I used air). The other branch goes to the bottom of the test container, and ends in a fine lube, which is designed to support one end of the organ with the other end being supported by another loop which hangs by a wire from the recording device.

Recording device. With a celuloid point.
Chromagraph covered with smoked paper.
Thermometer placed in the bath.
Solutions. a) Ringer solution. Preparation:

Solution A

Sodium chloride	90 gms
Potassium chloride	4.2 gms
Calcium chloride	2.4 gms
Magnesium chloride	2 gms
Distilled water c.p.c.	1000 c.c.

Solution B

Sodium bicarbonate	5 gms
Glucose	10 gms
Water c.s.p.c.	1000 c.c.

100 cmc. of the A solution and 100 of the B solution are measured, the volume is filled to 1000 c.c. with recently distilled water. The solution thus prepared corresponds to the following formula:

Sodium Chloride	0.9
Potassium chloride	0.42
Calcium chloride	0.24
Magnesium chloride	0.2
Sodium bicarbonate	0.5
Glucose	0.1
Distilled water c.s.p.c.	1000 c.c.

Spasmodic solutions used.
Pituitrine solution (SHarp Dome) 1 l U.V.
Ergotamine solution (Sandox Neoginergene) 1 c.c. 0.2 mgs.
Barium chloride solution 1 c.c. 10 mgs
Histamine solution at 1 x 3,750,000.

Preparation of the organ. After the animal has been sacrificed, it is drained of blood by placing the lower extremeties in an upward position, immediately the thoraxic cavity is opened. The intestinal mass is separated, beneath the uterus appears which is extracted with care.

The uterus as well as a portion of the intestine is placed in a Container of Ringer solution, and in a refrigerator so as to conserve them without their losing their sensitivity.

Factors which must not vary. The continual bubbling of air assures the constant sensitivity of the organ. The temperature must be maintained constantly between 37 and 38°C.

Tests. After having suspended the uterine piece in the test container, the horizontal position of the recording device is adjusted, by putting it in contact with the recording drop, assuring that the celuloid point lightly touches the smoked paper. 1 c.c. of pituitrine solution is added around the organ. Having caused the contraction of the organ by the addition of pituitrine, 1 c.c. of beta glycerophosphate solution at 50% is added. Shortly after a complete relaxation of paralysis of the organ is recorded. New amounts of sodium beta glycerophosphate are added; the organ does not respond to the addition of the pituitrine until it has been completely washed. While using ergotamine tartrate as a contractant, a similar action is observed. The addition of 0.2 mg. of the same causes a few seconds later the contraction of the uterine piece, while the addition of 1 c.c. sodiuma beta glycerophosphate at 50% reduces more relaxation. The addition of an additional amount of contractant remains without response. Washing of the organ as in the preceeding case causes it to recover its normal contraction capacity.

While using histamine in concentration of 1:3,750,000 as contractant the relaxing action of sodium beta glycerophosphate is observed later. The addition of new amounts creates contractions but of lesser intensity than normal. If the organ is washed, the addition of the same quantity (first) creates energetic contractions.

Finally while using 10 mg. of barium chloride it is observed from the addition of 1 c.c. of sodium beta glycerophosphate at 50% causes the relaxation of the contracted uterus. The addition of new amounts as in the preceding case causes responses that are much lower than the first. ON washing the organ the condition of barium chloride once again provokes contraction.

These experiments were repeated for a second time with the same results. On trying to relax the isolated guinea pig intestine, made spasmodic with barium chloride and histamine by the addition of sodium beta glycerophosphate, in the same concentration as before, no result was obtained, but the most sometimes it entenuates a bit contractions.

On the other hand, glycerine is added to the intestine in spasm, it causes a deep contraction similar to that of sodium beta glycerophosphate on the uterus.

The capacity of the bath in earlier experiments has been 100 c.c. The proper functioning of the apparatus was proved previously.

Conclusions

1. Sodium beta glycerophosphate is a medicine with antispasmodic and analeptic function on the uterus.

2. The antispasmodic action on the uterus in the isolated uterus is shown by succeeding in destroying the action of spasm producing substances

3. The antispasmodic action of sodium beta glycerophosphate is so intense that the contracting action of histamine and barium chloride.

4. The antispasmodic action of sodium beta glycerophosphate is specific for the uterine fiber, because it does not suppress the spasms caused in the intestine and it is lacking in action on arterial pressure.

5. The study of sodium beta glycerophosphate shows its action with relatively large amounts while it is perfectly tolerated by the organism. Animals can receive intravenously up to 1 gram of sodium beta glycerophosphate per kilo of body weight without displaying signs of intolerance. In obstretics Botella Llusia and Garcia Funcata have used amounts of 15 grams daily, without observing any toxic manifestations.

6. Sodium beta glycerophosphate may be considered as the best of known antispasmodic agents.

7. The respiratory analeptic action of sodium beta glycerophosphate is no less important; it may be used to this end in cases of intoxication by gases, narcotics, respiratory attacks and in asphyxia of the newborn.

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Estudio Farmacológico Experimental de la Acción Utero Espasmolítica del Beta Glicerofosfato de Sodio

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El estudio farmacológico del Beta Glicerofosfato de sodio, lo inició el profesor B. Lorenzo Velazquez, de Madrid en el año 1944, demostrando que es espasmolítico uterino y analéptico, como lo comprobaron posteriormente, Pérez Carnicero y García del Jalón en 1946, en la Cátedra Madrileña de Farmacología.

Esta acción útero espasmolítica demostrada al principio experimentalmente, fué puesta en evidencia obstétrica por Botella Llusia.

Desde entonces este fármaco ha concitado la atención de algunas Obstras y farmacólogos españoles, que han confirmado su acción espasmolítica uterina específica.

En Sur América han estudiado el tema Daniel Carril en Chile y E. Montragail y Oliveiro Pinto en Brasil, desde el punto de vista tocológico.

En el Perú, no se ha ensayado aún, siendo el Profesor Dr. Carlos A. Bambarén, el único que lo estudia en la Cátedra de Farmacología de la Facultad de Farmacia de Lima.

GLICEROFOSFATO DE SODIO Y SUS PROPIEDADES FARMACOLOGICAS

El estudio de las propiedades farmacológicas del Glicerofosfato de sodio comienza con los trabajos de **B. Lorenzo Velázquez** y sus colaboradores García del Jalón y Pérez Carnicero, en España, el año 1944, como una consecuencia de investigar la acción de la Glicerina sobre la musculatura lisa.

Estos autores trataron de explicar la acción calmante que en los cólicos hepáticos y nefríticos produce la inyección intravenosa de Glicerina, y comprobaron que la Glicerina es in vitro un antiespasmódico de todos los órganos de musculatura lisa. Este efecto es atribuído a la función polialcohólica, ya que aún la función monoalcohólica engrasada a cuerpos de cierto peso molecular, y de cierta estructura química posee propiedades relajadoras de la fibra lisa contracturada, como **Pérez Cirera** en 1938 demostró para el Alcohol octílico, y mas tarde **García del Jalón** (1944) demostró para el Fenil propílico, siendo **B. Lorenzo Velásquez** y **Pérez Carnicero** los que demostraron la acción espasmolítica de los polialcoholes **(21)**.

De los alcoholes ensayados en Farmacología el menos tóxico es la Glicerina, pero su aplicación resultó un poco peligrosa por la hemólisis que produce. Ello indujo a **Lorenzo Velázquez** y colaboradores a estudiar otros cuerpos, que llevando en su molécula la Glicerina tuviesen las acciones de este Propano triol sin efectos tóxicos, estudian los Glicerofosfatos, llegando a descubrir que el Glicerofosfato de sodio reduce notablemente las contracciones del músculo uterino espasmolizado, acción que no ejerce en otros órganos de musculatura lisa.

De los dos isómeros, el derivado Beta resultó con actividad mucho mas acentuada que el derivado Alfa (20).

De los Glicerofosfatos ensayados por dichos autores solamente el de Sodio y Magnesio se comportan como espasmolíticos, en tanto, que, los de Calcio, Zinc, Fierro y Manganeso, aumentan los espasmos, y contracciones; en éstos el cation respectivo predomina sobre el grupo Glicerofosfático.

El Glicerofosfato de magnesio a igualdad de dosis con el Glicerofosfato de sodio resulta mas activo (por que a la acción del radical Glicerofosfórico se suma la intensa espasmólisis producida por el ión Magnesio) pero su acción no es específica para la fibra uterina, y además, suprime los movimientos normales del órgano al igual que los espasmos, y por otra parte lleva consigo la acción toxica del ión Magnesio. (20 (22).

La acción espasmolítica del Beta glicerofosfato de sodio la estudió mas tarde, en 1947 **L. Cuny** y **D. Quivy** (5) en Francia, comparándola con la de la Glicerina y Alfa glicerofosfato de sodio, llegando a las mismas conclusiones que las del profesor **Lorenzo Velázquez.**

En los ensayos efectuados en la clínica obstétrica, en relación con su actividad útero paralizante, observaron los investigadores que los niños cuyas madres fueron tratadas con Glicerofosfato de sodio, presentaban aumento considerable de la respiración.

Este hecho suscitó interés para estudiar experimentalmente su acción sobre los centros respiratorios, **Pérez Carnicero, García del Jalón,** y **Urtasum** (12) encontraron que la administración intravenosa de esta sal, producía en los centros respiratorios de animales intoxicados con anhidrido carbónico, un aumento considerable de la amplitud respiratoria. Este aumento en la amplitud y la frecuencia, es mas intenso cuando la inyección se realiza por vía sub-occipital, obrando en este caso sobre la presión arterial que disminuye.

Es de hacer notar aquí, por estar relacionado con el mecanismo de acción del Glicerofosfato de sodio, que la Glicerina en las mismas condiciones y concentraciones, aparte de su acción hipotensora, no produce ningún efecto.

En 1946 **Lorenzo Velázquez, Félix Saenz Sánchez** y **F. Javier de Ello** (17) tratando de investigar el mecanismo de la acción del Glicerofosfato de sodio, estudiaron las modificaciones de la actividad fosfatásica del suero sanguíneo por la administración intravenosa del Beta glicerofosfato de sodio, en perros llegando a las conclusiones siguientes:

1.—El Beta glicerofosfato de sodio inyectado endovenosamente en el perro, hace disminuir grandemente la actividad fosfatásica del suero sanguíneo.

2.—La recuperación de esta actividad se hace entre 1 y 24 horas después de la administración.

De lo expuesto se deduce que las principales acciones farmacológicas son:

b).—Acción útero espasmolítica.
a).—Acción analéptica respiratoria.

ACCION UTERO ESPASMOLITICA DEL BETA GLICEROFOSFATO DE SODIO

El útero es uno de los órganos de musculatura lisa, cuya motilidad está sometida por una parte a la influencia del Sistema Nervioso Vegetativo, y por otra parte posee en su seno la causa de su propia motilidad. (16)

Fisiológicamente, el útero, lo mismo que otros órganos de musculatura lisa, está intensamente inhibido, y solo se modifica en conexión con su función fisiológica.

El empleo de sustancias que modifican la contractibilidad uterina produce distintos resultados según el período en que se administran (9) (18).

Este órgano presenta hipotonía o hipertonía recurriéndose en estos casos a oecitócicos o espasmolíticos (18).

Las sustancias que actúan sobre el útero, aumentando su motilidad, son los oecitócicos, mientras que las que disminuyen el tono del útero, reduciendo la frecuencia de sus contracciones, y aminorando en amplitud, son los espasmolíticos (9). En este último grupo de sustancias se considera al Beta Glicerofosfato de sodio por las excelentes acciones que posee demostradas en la clínica y en la experimentación farmacológica.

Todo buen espasmolítico debe reunir las siguientes condiciones: (12)

1.—Producir una rápida y enérgica sedación uterina.

2.—Que no inhiba las contracciones rítmicas o espontáneas, ni impida la reacción del útero a las drogas espasmódicas.

3.—Que no cause efectos colaterales ni indeseables en otros órganos o aparatos.

4.—Debe ser inofensivo para la madre y el niño, a mas de responder por su integridad a la labor fisiológica del parto (18).

La tercera condición es la mas difícil de encontrar, por cuanto la mayoría de los fármacos que relajan la fibra lisa del útero, se comportan análogamente, sobre el sistema muscular, del intestino, de los vasos, y aún provocan otras acciones tóxicas (3) (9) (13).

El Beta glicerofosfato de sodio, reune excelentemente estas cualidades, en particular la tercera, tan difícil de encontrar en los fármacos espasmolíticos. Por estas propiedades, merece conceptuarse como el mejor de los espasmolíticos uterinos hasta ahora conocidos, exceptuando la Lutefina que por ser menos extraña al organismo, mejor dicho mas fisiológica, es mas tolerado por él.

El Glicerofosfato de sodio Beta constituye un espasmolítico uterino de acción puramente sintomática, obra suprimiendo los espasmos; aunque nuevo todavía, sin embargo, los ensayos clínicos efectuados son favorables a su aplicación.

Aplicación del Beta Glicerofosfato de Sodio en Obstetricia.—El Beta Glicerofosfato de sodio por su gran papel útero espasmolítico ha encontrado múltiples aplicaciones en obstetricia. **Botella Lusic** y **García Funcasta** fueron los primeros que lo ensayaron y sentaron las bases para su uso, y las dosis en que debe utilizarse en cada caso.

Se ha empleado en:

1.—**Aborto incipiente.**

2.—**Prevención del aborto y parto prematuro,** tras de traumatismos quirúrgicos.

3.—**Tratamiento de parto prematuro.**

En estos casos el Beta Glicerofosfato de sodio constituye medicamento sintomático, pero su eficacia supera a la de los tratamientos etiogénicos, (Luteína, Vit. E.) (1) (2) (4) (10) (14).

Se han observado algunos fracasos, que los autores han atribuído a distintas causas, tales como indicación incorrecta, o dosis insuficientes, haciendo notar especialmente que la medicación fracasó cuando se trataba de mujeres con antecedentes neuríticos. **Botella Llusia y García Funcasta** y **Roncales Cativiela** (2) (14), proponen asociar el tratamiento con la Vitamina B^1 y en algunos casos con Progestenona y el Tokoferol.

Mecanismo de acción del Beta Glicerofosfato de Sodio.—El mecanismo de acción del Beta Glicerofosfato de sodio, está supeditado a meras hipótesis de trabajo.

Destaca sobremanera la acción úterotropa de este fármaco, de tal manera que así como la Glicerina se comporta como espasmolítico de todos los músculos de fibra lisa, el Beta glicerofosfato de sodio solamente relaja frente a los espasmos del órgano uterino.

Esta acción específica según **Lorenzo Velázquez** (19) parece relacionarse con la fijación del compuesto glicerofosfórico en la fibra lisa del útero, **en íntima relación con el metabolismo especial fosforado de la fibra uterina,** sobre todo en estado grávido. El útero en estas condiciones muestra un tropismo especial para la molécula glicerofosfórica en su sal sódica (catión indiferente).

Este tropismo de puro matiz metabólico, se hace a travez del radical fosfórico y actuando sobre el Fosfato de Adenosina, necesaría la acción de la Colinesterasa para la génesis de la Acetilcolina. Apoya esta suposición el hecho de que el mismo Fosfato sódico refuerce el trabajo muscular, del útero **(Embden Deuticke)** (1941).

Por consiguiente el Beta glicerofosfato de sodio aumentaría las contracciones musculares pero, es que enseguida la fijación específica sobre el útero de la molécula glicerofosfórica por el radical fosfato en entonces cuando la función dialcohólica primaria produce la sedación o espasmólisis, función general a los alcoholes. Para todo ello y en especial para la fijación del grupo fosfático se necesita de cierto nivel de Pirofosfato de Aneurina, lo que explica porque falla la medicación en avitaminosis B^1.

Algo hay en el útero y no en otros músculos lisos, que explica el tropismo y espasmolisis uterina específica por este fármaco, sea por el metabolismo especial fosforado para la acetilcolinogénesis, mas activa en el útero grávido, sea en conexión con el ciclo de Meyerhoff del metabolismo hidrocarbonado, en el que aparece como intermediario el glicerofosfato, o sea en fin, por una acción especial enzimática en el útero, que disgregue específicamente el Beta glicerofosfato de sodio, dejando el polialcohol en libertad con la consiguiente espasmolisis.

En cuanto al mecanismo de acción sobre los centros respiratorios, la diferencia de acción con la glicerina, ya sobre la respiración o ya sobre la presión sanguínea, permite encontrar un punto de partida para establecer el posible mecanismo de acción en la favorable acción de la molécula glicerofosfórica.

Si la inyección de Glicerina y la inyección de glicerofosfato sódico son separadamente inactivos, y la molécula glicerofosfórica total resulta de marcada acción farmacológica, es posible que un doble mecanismo intervenga en esta acción. La función dialcohólica que queda libre del resto Glicérico, no explica el estímulo, pero si la fijación del glicerofosfato en los centros nerviosos (respiratorios y vasomotores) una vez fijada esta molécula el radical fosfórico activa la acetilcolinogénesis, en este sentido podría explicarse el marcado estímulo sobre tales centros y en forma específica sobre los respiratorios.

Desde los estudios de Nachmanson y colaboradores (1941-1943) se sa be que la colinesterasa necesita para su acción formadora de Acetilcolina la presencia de Trifosfato de Adenosina, en la recuperación de él interviene el desdoblamiento de la fosfocreatina. El ra-

dical fosforado o fosfatado del glicerofosfato favorece este ciclo metabólico en la acetilcolinogénesis una vez fijada la molécula glicerofosfórica a los centros por los dos alcoholes libres del radical glicérico. Esto entra dentro de lo posible y será muy difícil demostrarlo en tanto no se conozca a fondo y con todo detalle el metabolismo de la acetilcolinogénesis.

Investigaciones efectuadas.—He estudiado experimentalmente la acción útero espasmolítica del Beta Glicerofosfato de sodio, sobre el órgano aislado de cobaya, y el efecto que produce el intestino aislado.

Técnica.— La acción espasmolítica del Beta glicerofosfato de sodio se puede comprobar:

1.—Por disminución o supresión de la respuesta del órgano a las drogas que aumentan su actividad por adición previa del glicerofosfato al líquido nutricio.

2.—Por el efecto que produce su adición sobre el órgano previamente espasmódico.

He utilizado este último método por considerar mas apropiado para apreciar las modificaciones de la actividad uterina y por que la producción previa del estado espasmódico que se manifiesta por el aumento de las contracciones del órgano, es como una comprobación de sensibilidad del órgano a las sustancias espasmódicas, necesarias en este caso para comprobar la acción relajante de este fármaco.

Dispositivo.— Está constituído del siguiente sistema:

Baño María.— Es un recipiente metálico de varios litros de capacidad con regulación termoeléctrica destinada a mantener una temperatura constante.

Recipiente de ensayo.— Es un cilindro de vidrio que por extremidad inferior termina en un tubo delgado. Por intermedio de un tubo de jebe está en comunicación con un serpentín de vidrio introducido en el interior del baño.

El aprovisionamiento y desagüe del recipiente de ensayo con solución Ringer, se verifica por este serpentín y por intermedio de una llave de doble vía. Por su parte superior amplia, recibe la droga por ensayar.

Burbujeador de Oxígeno.—Está formado por un tubo de vidrio doblado en U, uno de los extremos está en comunicación con un generador de oxígeno, pudiendo utilizarse aire (En mis experiencias he utilizado aire). La otra rama va al fondo del recipiente de ensayo, y termina en un gancho fino, está distinado a sostener un extremo del órgano estando el otro extremo sostenido por otro gancho que por un hilo pende de la palanca inscriptora.

Palanca inscriptora.— Con una punta de celuloide.
Quimógrafo recubierto de papel ahumado.
Termómetro introducido en el interior del baño.
Soluciones.— a) Solución Ringer. Preparación:

Solución A			Solución B		
Cloruro de sodio	90	gms.	Bicarbonato de sodio	5	gms.
Cloruro de potasio	4.2	„	Glucosa	10	„
Cloruro de calcio	2.4	„	Agua c.s.p.c.	1000	c.c.
Cloruro de magnesio	2	„			
Agua destil. c.p.c.	1000	c.c.			

Se mide 100 c.c. de la solución A y 100 de la sol. B, se completa el volumen a 1000 c.c. con agua recientemente destilada. La solución así preparada responde a la siguiente fórmula:

Cloruro de sodio	0.9	
Cloruro de potasio	0.42	
Cloruro de calcio	0.24	
Cloruro de magnesio	0.2	
Bicarbonato de sodio	0.5	
Glucosa	0.1	
Agua destil. c.s.p.c.	1000	c.c.

Soluciones espasmódicas utilizadas.—
Solución de Pituitrina (Sharp Dome) 1 1 U.V.

Solución de Ergotamina (Neoginergeno Sandoz) 1 c.c. 0.2 mgs.
Solución de Cloruro de bario 1 c.c. 10 mgs.
Solución de Histamina al 1 x 3,750,000.

Preparación del órgano.— Sacrificado el animal se desangra colocando las extremidades inferiores hacia arriba, enseguida se abre la cavidad toráxica: Se separa la masa intestinal, en el fondo aparece el útero que se extrae con cuidado.

Tanto el útero como una porción del intestino se colocan en un vaso con solución Ringer, y en refrigeradora para conservarlas sin que pierdan su sensibilidad.

Factores que no deben variar.— El continuo burbujear del aire asegura la constante sensibilidad del órgano. La temperatura debe mantenerse constantemente entre 37 a 38º C.

Ensayo. - Suspendido el trozo uterino en el recipiente de ensayo, se regula la horizontalidad de la palanca inscriptora, poniéndola en contacto con el tambor inscriptor, procurando que la punta de celuloide roce levemente el papel ahumado. Se agrega 1 c.c. de solución de Pituitrina al rededor del órgano. Provocada la contracción del órgano por la adición de Pituitrina, se añade 1 c.c. de solución de Beta glicerofosfato al 50%. Poco después se registra una completa relajación y parálisis del órgano. Se adicionan nuevas dosis del Beta Glicerofosfato de sodio, el órgano no responde a la adición de Pituitrina hasta que se lava perfectamente. Utilizando como contracturante Tartrato de Ergotamina, se observa una acción semejante. La adición de 0.2 de mg. de la misma provoca pocos segundos después la contracción del trozo uterino, la adición de 1 c.c. de beta glicerofosfato de sodio al 50% produce relajación. La adición de nueva cantidad de contracturante queda sin respuesta. El lavado del órgano como en el caso anterior le hace recuperar su capacidad normal de contracción.

Empleando Histamina en concentración de 1:3.750.000 como contracturante se observa después la acción relajante del Beta glicerofosfato de sodio. La adición de nuevas dosis origina contracciones pero de intensidad inferior a las normales. Si el órgano se

lava la adición de la misma cantidad (primera) provoca contracción enérgica.

Finalmente utilizando Cloruro de bario 10 mg. se observa como la adición de 1 c.c. de Beta glicerofosfato de sodio al 50% provoca la relajación del útero contracturado. La adición de nuevas dosis como en el caso anterior provoca respuestas pero muy debajo de la primera. Al lavar el órgano la adición de Cloruro de bario provoca la contracción nuevamente.

Estas experiencias fueron repetidas por segunda vez con los mismos resultados.

Al tratar de relajar el intestino aislado de cobaya, espasmodizado con Cloruro de bario e Histamina por la adición de Beta Glicerofosfato de sodio, en la misma concentración anterior, no se obtuvo ningún resultado, cuando mas algunas veces atenúa un poco las contracciones.

En cambio, si sobre el intestino espasmodicado se añade Glicerina provoca una descontracturación semejante a la del Beta Glicerofosfato de sodio sobre el útero.

Nota.— La capacidad del baño en todas las experiencias ha sido de 100 c.c. El buen funcionamiento del aparato ha sido comprobado previamente.

CONCLUSIONES

1.—El Beta Glicerofosfato de sodio es un fármaco de acción útero espasmolítica y analéptica.

2.—La acción útero espamolítica en el útero aislado se demuestra al conseguir destruir la acción de las sustancias espasmódicas.

3.—La acción espasmolítica del Beta glicerofosfato de sodio es tan intensa que contrarresta la acción contracturante de la Histamina y el Cloruro de bario.

4.—La acción espasmolítica del Beta glicerofosfato de sodio es específica para la fibra uterina, por que no suprime los espasmos provocados en el intestino y carece de acción sobre la presión arterial.

5.—El Beta Glicerofosfato de sodio manifiesta su acción a dosis relativamente grandes, siendo perfectamente tolerado por el organismo. Los animales pueden recibir por vía intravenosa hasta 1 gramo de Beta Glicerofosfato de sodio por kilo de peso sin presentar signos de intolerancia. En obstetricia **Botella Llusia** y **García Funcasta** han utilizado dosis de 15 gramos diarios, sin observar manifestaciones tóxicas.

6.—Puede considerarse al Beta Glicerofosfato de sodio como el mejor de los espasmolíticos conocidos.

7.—La acción analéptica respiratoria del Beta Glicerofosfato de sodio no es menos importante, puede utilizarse con este fin en casos de intoxicaciones por gases, narcóticos, síncopes respiratorias y en la asfixia de los recién nacidos.

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Effect of Phosphoric Acid Esters on Fracture Healing.*

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The erythrocytes contain an abundance of phosphoric esters hydrolyzable by phosphatase,[1] but the plasma contains only 0.2-0.5 mg phosphorus per 100 cc[2] in the form of phosphatase hydrolyzable esters. The phosphatase activity of a fracture callus increases rapidly beginning 3-4 days after the injury.[3, 4, 5] Although the site

* This work was supported by Grant 557 of the Committee on Scientific Research of the American Medical Association.

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of an old fibrous non-united fracture may exhibit low phosphatase activity[3] and infected fractures contain somewhat reduced amounts of this enzyme[6] there is no evidence that defective calcification of bone or delay in fracture healing is primarily a result of a quantitative deficiency of this enzyme. It appears possible that one of the factors limiting the speed of the calcification process is the low content of plasma with respect to the substrate of bone phosphatase and this consideration suggested that some cases of delayed or nonunion of fractures might be so explained. It seemed desirable, therefore, to investigate the effect of the intravenous injection of sodium beta-glycerophosphate on the healing of fresh fractures.

The right radius of rabbits, weighing between 4.25 and 4.75 lb. was fractured while the animals were anesthetized. The fractures were produced by grasping the middle of the shaft of the bone between two adjacent and parallel Kocher forceps and exerting steady rotation of the forceps in opposite direction until the bone was heard to break. A few animals, in which breaks of both the radius and ulna were produced, or which displayed marked bruising of the skin, were discarded. Roentgenological examination showed that the fractures usually were transverse and that the fragments were in good alignment with little comminution of the bone. The undamaged ulna served as an effective splint and the animals were able to use the limb upon recovery from the anesthesia.

Twenty-seven animals of the experimental group received 6-14 daily injections, by ear vein, of 0.1 g sodium beta-glycerophosphate in 4 cc of solution. Twenty-two animals with similar fractures served as controls and received a like number of injections of 4 cc of physiological saline. All animals were sacrificed on the day following the last injection. Roentgenograms of the limbs, taken before and after removal of the soft tissues, did not reveal any marked differences except for a larger callus about the fracture of the bones of the animals treated with sodium beta-glycerophosphate. However, the fracture callus of the animals receiving 8-14 treatments with sodium beta-glycerophosphate was larger and more firm to palpation than that of the control animals. After the bones were cleaned, even though some of the callus was lost when the periosteum was removed, the healing of the fracture appeared to have progressed further in the animals treated with glycerophosphate. This judgment was based upon the observation that the hard callus of the treated animals was larger and the fracture defect was more

[6] Drummond, D. H., Tollman, J. P., Bisgard, J. D., and Ross, D., *Arch. Surg.*, 1940, 40, 49.

nearly bridged over in the treated animals than in the controls. A more objective means of estimating the degree of healing of fractures would be desirable. However, the observations indicated that in the majority of cases the drug exerted a positive effect even though the fractures with which we were dealing normally progress to rapid healing. Four determinations of serum inorganic phosphate and calcium, made at regular intervals during the course of 12 daily injections of 0.1 g of glycerophosphate, showed no consistent differences from the results obtained with untreated animals.

Sodium beta-glycerophosphate, when given by intravenous injection over a long time or in large doses, is not toxic. The liver, intestine, urinary bladder, kidney, heart and aorta of a rabbit which received 55 daily injections of 0.1 g of the drug were normal grossly and microscopically. Similar results were obtained with 3 rabbits following 6 daily intravenous injections of 1.0 g of the substance. No symptoms were produced following 26 daily subcutaneous injections of 0.1 g of sodium beta-glycerophosphate into a rabbit and no pathological findings were noted at autopsy. A 17 kg dog received 10 daily intravenous injections of 10 g of the substance in 40 cc of solution. No ill effects were produced and when the animal was sacrificed, 2 months after the last injection, and no pathological alterations of any organ were observed. Large doses (12 to 24 cc of a solution containing 0.25 g of the substance per cc) are fatal in 2-5 days when given daily to rabbits by intraperitoneal injection.

Since sodium beta-glycerophosphate is innoxious in a wide range of dosage it should be safe to administer it to the human in order to determine its effect on those fractures which heal at a rate slower than usual.

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EFFECT OF GLYCERINE AND GLYCEROPHOSPHATE ON THE NATURE OF EXPERIMENTAL HYPERCHOLESTERINEMIA IN RABBITS

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As is well-known, long-term administration of cholesterol with food leads to the disruption of matter exchange, being manifested in atherosclerotic changes in blood vessels and organs.

Therefore exogenic cholesterol introduced in excess must be considered a toxic substance. In such situations, the liver, as a barrier organ, must assume a major role.

In studying the barrier function of the liver, the method of overload of the substance studied is employed.

However the study of the toxic action of cholesterol on the liver is fraught with difficulties, for cholesterol is barely soluble in water and can only be administered by mouth. This does not give complete confidence that all the cholesterol given in the food will be absorbed. On the other hand, a single overload does not evoke hypercholesterinemia.

In order to cause feeding hypercholesterinemia, long-term administration of cholesterol with food is necessary, since its level in the blood is maintained with ahigh constancy.

It is possible to get around these difficulties, however, if cholesterol is introduced with feed which is easily eaten up by rabbits and if it is added to the food over several days.

Desiring to study the liver's role in rendering cholesterol harmless and the effect on this function of glycerine and glycerophosphate, the following method was employed:

Three groups of adult rabbits were chosen. One, the control (29 animals), received cholesterol daily with their feed for seven days in doses of 0.5 g/kg body weight per day. The second group - 9 rabbits - received supplementally for the same length of time 50 mg/kg of glycerine with their feed. The third group of animals (11 rabbits) was given, instead of glycerine, 0.1 g/kg of calcium glycerophosphate. Cholesterol with the supplments was given together with with 50 g of ground carrot. Blood for the study was taken on an empty stomach in the morning prior to overload, and thereafter 1, 7-8, 11-13, and 16-18 days after the end of overload. Determination of cholesterol and its esters was done by the digitonin method.

Results of the Experiment

As is evident from the data presented in Table 1 (see the table below), the normal levels of total, esterified, and free cholesterol in the blood of the control animals, according to our data, are 65 + or - 2, 39 + or - 2, and 26 + or - 2 mg %, respectively. Twenty-four hours after the cessation oc cholesterol overload, the level of all the fractions of cholesterol increased: total - to 170 + or - 10 mg %, esterified - to 113 + or - 10 mg %, and free - to 56 + or - 5 mg %. At 7-8 days, the cholesterol level and that of its fractions gradually decreased, although it continued to remain at a higher level at 16-18 days.

TABLE 1. LEVEL OF TOTAL, ESTERIFIED, AND FREE CHOLESTEROL IN THE BLOOD OF RABBITS, IN MG-% NORMAL ANIMALS, WITH THE ADDITION TO A CHOLESTEROL DIET OF GLYCERINE AND CALCIUM-GLYCEROPHOSPHATE (mean data)

Time of Blood Sample	Control or Exper.	Cholesterol Fraction: Total	Rel. Inc.*	Esters	Rel. Inc.	Free	Rel. Inc.	% Free Cholesterol
Before overl.	Control	65±2	1.0	39±2	1.0	26±2	1.0	40.0
1 day after o.l.	Control	170±10	2.6	113±10	2.9	56±5	2.1	32.9
	Glycer.	201±12	3.1	157±11	4.0	38±6	1.8	23.8
	Glyc.-PO_4	203±26	3.1	150±19	4.0	53±10	2.0	36.1
7-8 day after	Control	150±5	2.3	99±8	2.5	52±6	2.0	34.6
	Glycer.	170±14	2.6	117±14	3.2	54±4	2.0	31.1
	Glyc.-PO_4	250±16	4.0	196±13	5.0	62±8	2.3	24.0
11-13 days after	Control	123±7	1.9	78±5	2.0	47±5	1.6	36.5
	Glycer.	158±13	2.4	104±11	2.6	53±10	2.1	34.6
	Glyc.-PO_4	199±18	3.0	149±15	3.0	50±4	1.9	25.1
16-18 days after	Control	100±7	1.5	64±5	1.6	36±4	1.4	36.0
	Glycer.	138±14	2.1	88±11	2.2	50±6	1.9	35.5
	Glyc.-PO_4	155±10	2.3	114±17	2.9	36±4	1.3	26.4

* Relative increase of fraction means the ratio of the amount of cholesterol or its fraction to the original level prior to overload

At 7-8 days the quantity of total cholesterol was 150 + or - 5, of esterified cholesterol 99 + or - 8, and of free 52 + or - 6. Toward the end of the experiment, i.e. at 16-18 days, they were 100 + or - 7, 64 + or - 5, and 36 + or - 4 mg %, respectively.

From a comparison of the coefficients of free and associated cholesterol, it is evident that the relative increase in esterified cholesterol is significantly greater than that in free cholesterol. On the other hand, a greater persistence of free cholesterol is noted in the blood in comparison to its esters. In other words, the liver, in answer to cholesterol overload, renders it harmless by means on converting it into esters which evidently possess a less toxic effect.

Considering the level of total cholesterol in the blood with the addition of glycerine to food, it is possible to see that its content at all periods of study is higher than in the control group, although its increase is not statistically significant.

Esterified cholesterol yields clearer results. It is also increased in the blood in relation to the control at all periods of study after overload, and its increase is statistically significant at 1, 11-13, and 16-18 days. A relative increase in esters of cholesterol in the blood after cholesterol and glycerine overload is significantly greater than during only cholesterol overload. From this it is clear that it is a question of relatvie coefficients. So for 24 hours after cholesterol and glycerine overload, the coefficient equalled 4.0, i.e. the quantity of cholesterol esters increased four times in relation to the norm, at the same time for which the coefficient in the control was 2.9. This difference in coefficients remained significant even at 16-18 days.

Pertinent to the experiment, esterified cholesterol increased 2.2 times, while in the control it increased only 1.6 times.

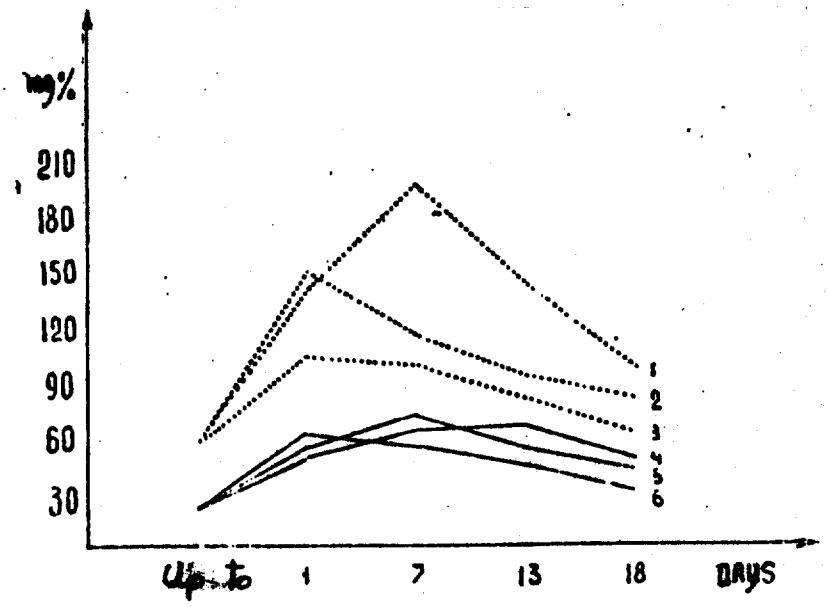
Regarding free cholesterol, its level differs little from the level in the control, but the relative increase after overload was somewhat less than in the control. Changes in level of esterified and free cholesterol are presented in Fig. 1.

In the experiments with a single load of cholesterol and glycerine, as in the controls with an overload of only cholesterol, it is noted that hypercholesterinemia occurs mainly due to cholesterol esters.

In the experimental animals, percentage of free cholesterol in the blood is less than in the controls. Thus after loading, at 24 hours, the quantity of free cholesterol was 23.8% and in the control it was 32.9%.

Finally, the effect of calcium glycerphopsphate on the nature of hypercholesterinemic curves was studied.

From the data presented in the table, it is evident that the total cholesterol content increases sharply in the presence of glycerophsophate in the food, in relation to the control, and this increase is observed not only at 24 hours but at 7-8 days after overload. This increase is not observed either in the control or in the glycerine experiment. The increase is statistically significant. However the greatest change is observed on the part of cholesterol esters. Their relative increase at 1, 7-8, and 16-18 days were equal to 4.0, 5.0, 3.9, and 2.0 respectively (sic), and in the control were 2.9, 2.5, 2.0, and 1.6 (sic) respectively.



Dynamics of cholesterol changes in the blood of rabbits during addition of glycerine and calcium glycerophsophate to a cholesterol diet (Control 3 and 6, glycerine 2 and 4, glycerophsophate 1 and 5; solid line - free cholesterol, dotted line - cholesterol esters)

Regarding the free cholesterol content in the blood, as with the addition of glycerine, it differed very little from the norm. Its content in relation to total cholesterol was significantly decreased due to cholesterol esters. This dependence is evident in Fig. 1, where variation in free and esterified cholesterol after glycerophsophate dosage are indicated in comparison with the control and glycerine in the same row.

On the graph the sharp increase in cholesterol esters in the blood is clearly evident under the influence of glycerine as well as a still higher level of calcium glycerophosphate.

Consideration of Results of the Experiments and Conclusions

Our studies, it seems to us, permit one to state that long-term introduction of exogenic cholesterol must be considered in this case not as a normal product, but as a toxic substance. We may speak of the nature of the hypercholesterinemic curves. Data from the literature and from generally-known facts concerning the increase in cholesterol level in the blood, after receiving large doses with food, show that in regulating the cholesterol level in the blood, the liver plays a most important, if not major, role. Thus Fridman, Bayers, and Rozenman found that six hours after oral administration of cholesterol to rats, the latter was found in Kipfer cells. These cells evidently are responsible for cholesterol inhibition in the liver, not allowing it to pass further into the general bloof circulation. With more extended oral administration of cholesterol in food, the liver begins to let it pass into the blood, evoking hypercholesterinemia.

As is evident from our data, regulation of the free cholesterol level in the blood takes place due to conversion of a large part of it into esters. In this process, the liver also assumes a non-final role.

Thus, our data have shown that oral administration of cholesterol to rabbits in the course of a week evokes hypercholesterinemia, due primarily to esterified cholesterol, at the same time as the amount of free cholesterol in the blood maintains constancy. The addition of glycerine and glycerophosphate simultaneously with cholesterol, practically speaking, increases only the level of ester-linked cholesterol, showing no notable effect on free cholesterol.

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ВЛИЯНИЕ ГЛИЦЕРИНА И ГЛИЦЕРОФОСФАТА НА ХАРАКТЕР ЭКСПЕРИМЕНТАЛЬНОЙ ГИПЕРХОЛЕСТЕРИНЕМИИ У КРОЛИКОВ

Т. В. БАБИНА и *И. В. СИДОРЕНКОВ*

Как хорошо известно, длительное введение кроликам холестерина с пищей приводит к нарушению обмена веществ, проявляющемуся в виде атеросклеротических изменений в сосудах и органах.

Поэтому в избытке вводимый экзогенный холестерин надо рассматривать как токсическое вещество. В этих условиях печени, как барьерному органу, должна принадлежать большая роль.

Для изучения барьерной функции печени применяется метод нагрузки исследуемого вещества.

Однако исследование токсического действия холестерина на печень сопряжено со значительными трудностями, ибо холестерин почти не растворим в воде и его можно вводить только перорально. А это не дает полной уверенности, что весь холестерин, даваемый с пищей, всасывается. С другой стороны однократная нагрузка не вызывает гиперхолестеринемии.

Для того, чтобы вызвать пищевую гиперхолестеринемию, нужно продолжительное введение холестерина с пищей, так как уровень его в крови поддерживается с большим постоянством.

Эти трудности однако можно обойти, если холестерин вводить с кормом, который легко поедается кроликами и добавлять его к пище несколько дней.

Желая изучить роль печени в обезвреживании холестерина и влияние на эту функцию глицерина и глицерофосфата нами была применена следующая методика:

Брали три группы взрослых кроликов. Одна контрольная, (29 животных) ежедневно получала с пищей холестерин в течение 7 дней по 0,5 г/кг веса тела в сутки. Вторая — (9 кроликов) дополнительно получала в течение того же срока с пищей

по 50 мг/кг глицерина. Третьей группе животных (11 кроликов) давали вместо глицерина по 0,1 г/кг глицерофосфата кальция. Холестерин с добавками давался вместе с 50 г тертой моркови. Кровь для исследования брали натощак утром до нагрузки, а затем через 1,7-8; 11-13; и 16-18 дней после прекращения нагрузки. Определение холестерина и его эфиров производилось дигитониновым методом.

Результаты исследования

Как видно из приведенных данных, в таблице 1 (см. таблицу ниже) нормальное содержание общего, этерифицированного и свободного холестерина в крови у контрольных животных, по нашим данным, равно соответственно 65±2; 39±2 и 26±±2 мг%. Через сутки после отмены холестериновой нагрузки, содержание всех фракций холестерина увеличивалось: общего до 170±10 мг%, этерифицированного до 113±10 мг% и свободного до 56±5 мг%. Через 7-8 дней уровень холестерина и его фракций постепенно снижается, хотя продолжает оставаться

Таблица 1

Содержание общего, эфиросвязанного и свободного холестерина в крови кроликов в мг% у нормальных животных при добавлении к холестериновой диете глицерина и глицерофосфата кальция
(средние данные)

Время взятия крови	Контр. или опыт	Фракции холестерина						
		общий	отн. повыш.*	эфиры	относит. повыш.	свобод.	относ. повыш.	% свод. х-на
До нагр.	Контр.	65 ± 2	1,0	39 ± 2	1,0	26 ± 2	1,0	40,0
После нагр. через день	Контр.	170 ± 10	2,6	113 ± 10	2,9	56 ± 5	2,1	32,9
	Глицерин	204 ± 12	3,1	157 ± 11	4,0	48 ± 6	1,8	23,8
	глицерофосфат	203 ± 26	3,1	150 ± 19	3,0	53 ± 10	2,0	36,1
7—8 дней	контр.	150 ± 5	2,3	99 ± 8	2,5	52 ± 6	2,0	34,6
	глицерин	170 ± 14	2,6	117 ± 14	3,2	54 ± 4	2,0	31,1
	глицерофосфат	258 ± 16	4,0	196 ± 13	5,0	62 ± 8	2,3	24,0
11—13 дней	контр.	123 ± 7	1,9	78 ± 5	2,0	47 ± 5	1,6	36,5
	глицерин	159 ± 13	2,4	104 ± 11	2,6	55 ± 10	2,1	34,6
	глицерофосфат	199 ± 18	3,0	149 ± 15	3,9	50 ± 4	1,9	25,1
16—18 дней	контр.	100 ± 7	1,5	64 ± 5	1,6	36 ± 4	1,4	36,0
	глицерин	138 ± 14	2,1	88 ± 11	2,2	50 ± 6	1,9	35,5
	глицерофосфат	155 ± 10	2,3	114 ± 17	2,9	38 ± 4	1,5	26,4

* Относительное повышение фракций означает — отношение найденного количества холестерина или его фракций к первоначальному уровню до нагрузки.

повышенным спустя 16-18 дней. Через 7-8 дней количество общего холестерина было равно 150±5, эфиросвязанного 99±8 и свободного 52±6. К концу опыта, т. е. через 16-18 дней, он соответственно равнялся 100±7; 64±5 и 36±4 мг%.

Из сравнения коэффициентов свободного и связанного холестерина видно, что относительное повышение эфиросвязанного холестерина значительно больше, чем свободного. С другой стороны, отмечается большее постоянство свободного холестерина в крови по сравнению с его эфирами. Другими словами, печень в ответ на нагрузку холестерином отвечает обезвреживанием его путем перевода в эфиры, которые, по-видимому, обладают менее выраженным токсическим действием.

Рассматривая уровень общего холестерина в крови при добавлении к пище глицерина можно видеть, что его содержание во все периоды исследования выше, чем в контроле, хотя это повышение статистически недостоверно.

Более отчетливые результаты дает эфиросвязанный холестерин. Он также повышен в опыте по отношению к контролю во все периоды исследования крови после нагрузки и это повышение статистически достоверно через 1,11-13 и 16-18 дней. Относительное повышение эфиров холестерина в крови после нагрузки холестерином и глицерином значительно больше, чем только при нагрузке одним холестерином. Об этом ясно говорят соответствующие коэффициенты. Так через сутки после нагрузки холестерином и глицерином коэффициент равен 4,0, т. е. количество эфиров холестерина увеличилось в 4 раза по сравнению с нормой, в то время как в контроле коэффициент равен 2,9. Эта разница в коэффициентах остается значительной еще через 16-18 дней.

Соответственно в опыте эфиросвязанный холестерин увеличен в 2,2 раза, в то время как в контроле только в 1,6 раза.

Что касается свободного холестерина, то его уровень мало отличается от уровня в контроле, а относительное повышение после нагрузки даже несколько меньше, чем в контроле. Изменения уровня этерифицированного и свободного холестерина, в одинаковом масштабе, представлены на рис. 1.

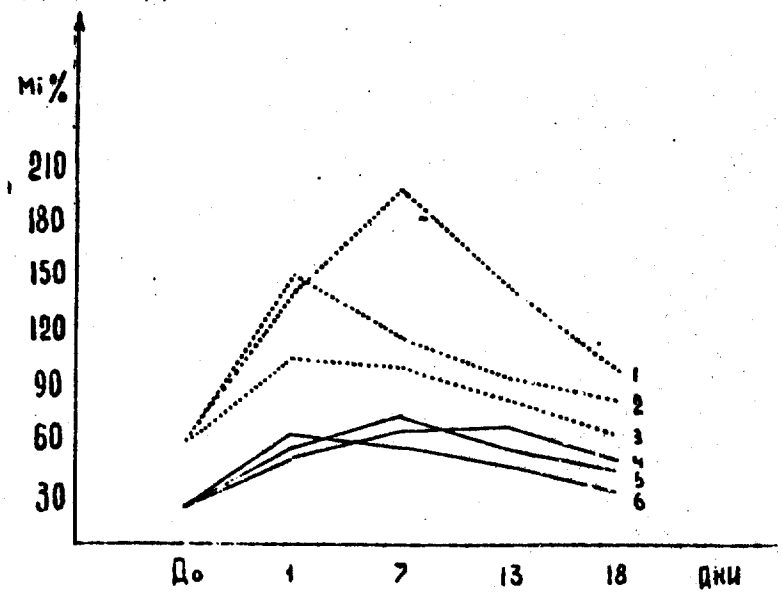
В опытах с одновременной нагрузкой холестерином и глицерином, также как и в контрольных с нагрузкой одним холестерином отмечается, что гиперхолестеринемия происходит, главным образом, за счет эфиров холестерина.

У опытных животных в связи с этим процент свободного холестерина в крови меньше, чем у контрольных. Так после нагрузки, через сутки, в опыте количество свободного холестерина составляет 23,8%, а в контроле 32,9%.

Наконец, было изучено влияние глицерофосфата кальция на характер холестеринемических кривых.

Из приведенных в таблице данных видно, что содержание общего холестерина в присутствии глицерофосфата в пище рез-

ко возрастает по отношению к контролю и это повышение наблюдается не только через сутки, но и спустя 7—8 дней после нагрузки, чего не наблюдается ни в контроле, ни в опыте с глицерином. Это повышение статистически достоверно. Однако наибольшие изменения наблюдаются со стороны эфиров холестерина. Относительное повышение их через 1,7-8, 16-18 дней равно соответственно в опыте 4,0; 5,0; 3,9 и 2,9, в контроле 2,9; 2,5; 2,0 и 1,6.



Динамика изменений холестерина в крови кроликов при добавлении к холестериновой диете глицерина и глицерофосфата кальция.
(Контроль 3 и 6, глицерин 2 и 4, глицерофосфат 1 и 5; ——— свободный холестерин, ... эфиры холестерина)

Что касается содержания свободного холестерина в крови, то он также, как и при добавлении глицерина практически не отличается от нормы. Его содержание по отношению к общему количеству холестерина значительно понижено за счет эфиров холестерина. Эта зависимость видна на рисунке 1, где показано изменение свободного, а также этерифицированного холестерина после нагрузки глицерофосфатом в сопоставлении с контролем и глицерином в равном масштабе.

На графике отчетливо видно резкое возрастание эфиров холестерина в крови под влиянием как глицерина, так и в еще большей степени глицерофосфата кальция.

Обсуждение результатов исследования и выводы

Наши исследования, как нам кажется, позволяют с определенностью высказаться за то, что длительно вводимый экзогенный холестерин, в этом случае надо рассматривать не как нормальный продукт, а как токсическое вещество. Об этом говорит характер гиперхолестеринемических кривых. Литературные данные и общеизвестный факт отсутствия повышения уровня холестерина в крови, после приема больших его доз с пищей, указывает на то, что в регуляции уровня холестерина в крови печень играет важнейшую, если не главную роль. Так Фридман, Байерс и Розенман нашли, что через 6 часов после перорального введения крысам холестерина последний обнаруживается в клетках Купфера. Эти клетки, по-видимому, и ответственны за задержку холестерина в печени не пропуская его дальше в общий круг кровообращения. При более длительном пероральном введении холестерина с пищей печень уже начинает пропускать его в кровь, что и вызывает гиперхолестеринемию.

Как видно из наших данных, имеет место регуляция уровня свободного холестерина в крови за счет перевода его большей части в эфиры. И в этом процессе печени также принадлежит не последняя роль.

Таким образом, наши данные показали, что пероральное введение холестерина кроликам в течение недели вызывает гиперхолестеринемию преимущественно за счет этерифицированного холестерина, в то время как количество свободного холестерина в крови поддерживается с большим постоянством. Добавление глицерина и глицерофосфата одновременно с холестерином практически повышают лишь уровень эфиросвязанного холестерина, не оказывая заметного влияния на свободный холестерин.

J. Lab. Clin. Med. 31: 1249-1250. 1946.

THE EFFECT OF GLYCEROPHOSPHATE ON BLOOD COAGULATION AND ON BLOOD CLOT RETRACTION

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THE anticoagulant effect of soluble glycerophosphates has been previously mentioned.[1] Since the solubility of calcium glycerophosphate, like the citrate, has a negative temperature coefficient, it seemed likely that calcium glycerophosphate would be poorly ionized and that glycerophosphate might inhibit coagulation by calcium ion depressant effect. This proved to be the case. Glycerophosphated plasma is readily coagulated by the addition of calcium chloride, sulfate, or dibasic phosphate, and whole glycerophosphated blood showed an unusual delay in spontaneous hemolysis on storage. Glycerophosphate found a limited application as an anticoagulant for blood specimens that had to be transported for long distances under unfavorable conditions prior to special serologic examination; however, since a final concentration of 2 per cent glycerophosphate in the plasma was found necessary for complete coagulation inhibition, the utility of this anticoagulant was limited by expense.

When less than 2 per cent glycerophosphate was added to freshly drawn blood, coagulation was delayed; however, after the advent of the latter, clot retraction appeared to be accelerated. Because there are clinical conditions in which clot retraction is deficient, this effect was further studied.

EXPERIMENTAL

Eastman's sodium glycerophosphate (52 per cent alpha) and Merck's beta preparation were used. Both gave essentially the same results. After dissolving the substance in water, the solutions were brought to pH 7.4 by the addition, dropwise, of concentrated HCl.

Blood was collected directly from the arm vein of human subjects by the insertion of a 3 inch, 20 gauge needle and allowed to flow directly down the glycerophosphated, solution-moistened wall of a graduated centrifuge tube containing measured quantities of 5, 10, or 20 per cent sodium glycerophosphate solution. During collection the centrifuge tube was continuously agitated by tapping the bottom to facilitate admixture with the anticoagulant solution, which, because of its high viscosity and specific gravity, otherwise tends to stratify at the bottom.

Coagulation time was measured by the Lee-White method[2] and clot retraction time by that of Macfarlane,[3] with the necessary modification that these determinations were made within the centrifuge tube into which the blood collection had been made.

Received for publication, June 19, 1946.

RESULTS

While a final concentration of 2 per cent or over of sodium glycerophosphate was found necessary to prevent coagulation indefinitely in most samples tested, in some, as little as 0.04 per cent would cause sensible inhibition. In other samples, 1 per cent glycerophosphate would delay coagulation for several hours. More constant, however, is the effect of subminimal anticoagulant quantities of sodium glycerophosphate on clot retraction time which is definitely diminished, as shown by representative experiments in Table I. Not amenable to quantitative representation is the fact that clots in glycerophosphated blood appear to shrink more rapidly and to attain a smaller final volume than in the control blood samples.

TABLE I. EFFECT OF SODIUM GLYCEROPHOSPHATE ON COAGULATION AND CLOT RETRACTION TIME

SAMPLE	GLYCEROPHOSPHATE (PER CENT)	CLOTTING TIME (MINUTES)	CLOT RETRACTION TIME (MINUTES)
Be	0.00	3.5	70
	0.25	5.0	40
	0.50	6.0	25
Myk	0.00	3.5	55
	0.10	4.5	35
	0.20	6.0	30
	0.50	9.0	20
Gi	0.00	5.0	80
	0.50	7.0	45
	1.00	8.5	30
	1.50	14.0	40
	2.00	No clot	

It might be mentioned that in the blood of a patient with terminal acute myeloid leucosis, with extensive purpura and a thrombocyte count of 28,000, the addition of 0.25 per cent sodium glycerophosphate did not induce clot retraction which was found to be absent both in the glycerophosphated and homologous control blood sample at the end of forty-eight hours. Also worthy of mention is the confirmation of the fact, previously noted, that blood samples containing completely anticoagulant concentrations of glycerophosphate, after a month of icebox storage, show barely a tinge of hemolysis.

SUMMARY

The addition of sodium glycerophosphate to shed blood in concentrations of 2 per cent or over completely prevents coagulation. Inhibition of coagulation is usually evinced by concentrations of 0.10 per cent and may be evinced in some samples by as little as 0.04 per cent. Concentrations which permit clotting cause an acceleration of clot retraction.

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Adsorption Analysis of Salts of Organic Acids

A preliminary report

by

Carl Olof Björling

State Pharmaceutical Laboratory, Stockholm

(Will be inserted in Collect. Pharm. Suec. IV 1949)

In recent years a new method for analysis of salts has been developed. Samuelson[1] filtered a solution of an inorganic salt through a column of a proton saturated cation exchanger. The anions passed through the column, but the cations —*e.g.* Li, Na, K, Ca, Ba, Cu, Pb, Fe, Al— were exchanged for hydrogen ions. The acid in the filtrate was titrated or otherwise determined. The cations withheld by the exchanger were eluted with hydrochloric acid and could be determined in the usual way. The yields were good and the time required for an analysis short. The method permits a separation of ions interfering with each other in analyses, *e.g.* phosphate from calcium. As very little is reported on salts of organic acids (however, compare 1) it seemed worth while to pay some attention to them. This paper is devoted to the analysis of sodium and calcium salts of some water soluble acids.

Procedure. The exchanger used was Amberlite IR-100 H from Nordiska Armaturfabrikerna, Linköping. To avoid swelling during the experiments the resin is kept in water for a day and then poured into a glass tube, 1 cm wide, to a height of 20 cm. When fixed vertically the column permits water to flow through it rather freely without suction. The rate of flow is regulated by a stop-cock at the lower end. The resin is washed with water until the washings are colourless and neutral to methyl red. Then 50 ml of hydrochloric acid 5M is filtered through the column (rate of flow: 5 ml per minute) and the excess of acid removed by washing with 200—500 ml of water (test with silver nitrate or methyl red). The column is then ready for the analysis.

Blank value. 100 ml of water run through the column and titrated with 1/10 normal sodium hydroxide (phenolphthalein) consumed 0,15 ml. With "equilibrium water" the value was 0,13 ml. Smaller quantities of water gave proportionally smaller blanks. All figures given below are corrected accordingly.

Capacity. The exchanging capacity of the column was tested in the following way. 20 ml of 1/10 molar sodium chloride solution was filtered through the column which was eluted completely with water (25 ml). The hydrochloric acid in the filtrate was titrated with 1/10 normal sodium hydroxide. Indicator: phenolphthalein. The process was repeated with further quantities of sodium chloride. Results see Table 1.

Table 1 Testing the Capacity of the Column

Millival Na Cl introduced	Millival HCl titrated
1,998	1,998
2,998	2,994
4,994	4,930
5,992	5,840

Thus, 2 millival could be used for the column.

RESULTS

1. *Salts, prepared extemporaneously.* 20 ml of one tenth normal solutions of some acids were titrated with one tenth normal sodium hydroxide, using phenolphthalein as the indicator. The titrated solutions were filtered (5 ml per minute) through the Amberlite column, and the acid re-liberated was eluted with water and titrated in the filtrate. All the chemicals used were duely tested for foreign anions. For results see Table 2.

Table 2. Acids, Titrated and Analysed

Acid	Millival found on		Yield %	Elution with ml
	direct titration	titration after Amberlite		
Tartaric acid	Free acid		99,9	50
	0,994	0,986	99,2	25
	0,994	0,992	99,8	50
	0,994	0,993	99,9	60
	2,004	2,001	99,9	50
	2,004	2,001	99,9	50
Citric acid	2,028	2,025	99,9	50
	2,028	2,024	99,8	50
Lactic acid	1,728	1,734	100,2	70
	1,728	1,728	100,0	70
Sulfosalicylic acid	1,536	1,534	99,9	50
	1,561	1,569	100,5	50

The direct determination of lactic acid was performed by the method given in the Swedish pharmacopoeia. For the "Amberlite analysis" the solution was heated on a water bath with 20 % excess of 1/10 normal sodium hydroxide for 5 minutes to destroy the anhydrides. The carbon dioxide of the air was excluded by means of a soda lime tube. After cooling the solution was filtered through the resin.

The exchange method failed with propionic and benzoic acids; for benzoic acid even in more dilute solution. The elution was slow and incomplete. Acetic acid has been tried with success by Samuelson[1].

2. *Calcium lactate*, conforming to the tests in the Ph. Svec. Ed. XI. It contained at most 1,2 % calcium lactyl lactate and 27,9 per cent water of crystallisation, the latter determined at 105° (theory 29,2 % H_2O).

About 0,3000 g dissolved in 10 ml of water was filtered through the column and eluted with 70 ml of water. Indicator: phenolphthalein.

Table 3. Calcium Lactate

	Per cent			
	Found	Mean	Theory	Yield
Ca, determ. as in Ph. Sv.	13,03; 13,13; 13,07	13,08	13,00	100,6
Acids, calculated as lactate ion. ..	57,3; 57,4	57,3	57,78	99,3

3. *Calcium gluconate*, conforming to the tests in the Ph. Svec. Ed. XI. The loss of water at 130° was 0,5 %. The water content according to the formula is 4 %.

About 0,4500 g dissolved in 20 ml of water. Elution with 70 ml of water. Indicator: phenolphthalein. Towards the end of the titration the red colour of the solution persisted for some seconds indicating a slow reaction between the sodium hydroxide and the titrand. However, there was no doubt as to where to fix the end point.

Table 4. Calcium Gluconate

	Per cent			
	Found	Mean	Theory	Yield
Ca, determ. as in Ph. Sv.	8,97; 9,01	8,99	8,94	100,6
Acids, calculated as gluconate ion	85,6; 86,6; 86,1	86,1	87,04	98,9

4. *Calcium glycero-phosphate*, conforming to the tests in the Ph. Svec. Ed. X. The loss of weight at 130° was 11,0 %. The water content according to the formula is 14,6 %.

About 0,2500 g dissolved in 20 ml of water. Elution with 25 ml of water. Indicator: phenolphthalein. A potentiometric titration ascertained that it was the correct indicator.

Table 5. Calcium Glycero-phosphate

	Per cent			
	Found	Mean	Theory	Yield
Calcium, titration to pH 3,5 ...	16,28; 16,45	16,37	16,28	100,6
Acids, calculated as $C_3H_7O_6P$ ...	69,8; 69,2	69,5	69,08	100,6

5. *Novalgin*, the sodium salt of amidopyrine methane-sulfonic acid (1 H_2O), free from chloride and sulfate. The loss of weight at 105° was 5,3 % (according to formula 5,1 % H_2O).

About 0,4000 g (1 millival) dissolved in 20 ml of water. The usual quantity 2 millivals, gave low yields (2—3 per cent loss). Elution with 50 ml of water. By a potentiometric titration it was ascertained that phenolphthalein was the correct indicator. The approximate content of amidopyrine was determined oxidimetrically with permanganate in alkaline solution by Schulek and Menyhárth's method.[8]

Table 6. Novalgin

		Per cent			
		Found	Mean	Theory	Yield
Bases, as Na (sulfate ash)		6,48; 6,59	6,54	6,55	99,8
Acids, as amidopyrine methane-sulfonium ion	With $KMnO_4$	89; 89		88,32	
	With Amberlite	87,5; 88,0	87,8		99,4

6. *Sodium menadiol sulfate*, Ph. Sv. (2-methyl-1,4-naphthylene-di-sodium-di-sulfate), a specimen of rather low quality: light brown colour; contained chloride and sulfate. About 0,4000 g dissolved in 20 ml of water. Elution with 50 ml of water. Indicator: phenolphthalein.

As it was found difficult to obtain a sulfate ash free from carbon, the sodium content was determined in the following way. After the elution with water, 50 ml of hydrochloric acid 5M was filtered through the column, thus eluting the sodium which was determined as Na_2SO_4. A blank was run.

The percentage of oxidizable hydroquinone derivative was determined with ceric sulfate by Canbäck and Ehrlén's method.[3]

Table 7. Sodium Menadiol Sulfate

	Per cent found, as $Na_2C_{11}H_8S_2$	Average
Acids	92,9; 92,7; 93,2; 93,0	93,0
Oxidizable	89,3; 89,8	89,6
Bases	93,5; 93,0	93,3

The result may be interpreted by the assumption that the substance contained 89,6 per cent sodium menadiol sulfate plus at most 1,4 per cent sodium sulfate or at most 1,1 per cent sodium chloride, the rest being water. This coincides with the outcome of the qualitative tests.

Summary

A method is given for the analysis of the sodium or calcium salts of some water soluble acids. A solution of the salt is filtered through a column of Amberlite IR-100 H, eluted with water, and titrated. The accuracy is good.

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2. *Wiesenberger, E.*, Mikrochemie ver. Mikrochim. Act. *30* 176, 1942.
3. *Schulek, E., Menyhárth, P.*, Z. analyt. Chem. *89* 426, 1932.

Bowen-'71
Bib No. 52

Caries Res. 5: 7–29 (1971)

Abstracts of Papers presented at the 17th ORCA Congress

July 14–17, 1970, Debrecen (Hungary)

The cariostatic effect of calcium glycerophosphate in monkeys (*M. irus*)

W. H. Bowen, Department of Dental Science, Royal College of Surgeons of England, London, England

A previous investigation (Bowen, Brit. Dent. J. *125*, 506–508, 1969) has shown that the inclusion of calcium glycerophosphate at a concentration of 1 % in sucrose effectively buffered acid production when applied to plaque. Because of this observation and the possibility that this substance could be bound in plaque by enzymes such as phosphatases and non-specific esterases, it was decided to investigate the effect of adding calcium glycerophosphate to the diet of monkeys on caries activity. 5 experimental and 6 control animals were fed a high carbohydrate diet. Both groups of animals received icing sugar 5 times daily but that fed to the experimental animals contained 1 % calcium glycerophosphate. After 22 months it was observed that the control animals have 47 carious lesions and the experimental animals 5 ($P_t<0.01$). All the control animals developed dental caries whereas 3 of the experimental animals remained caries-free. Total phosphate was higher in plaque from the animals receiving added phosphate than from those which weren't.

Animal experiments on the caries-reducing effect of glycerophosphate

C. H. Plathner and M. Winiker, Policlinic of Conservative Stomatology of the Department of Medicine, Humboldt University, Berlin, German Democratic Republic

In spite of the world-wide success of caries control by means of fluoride, the interest in other cariostatic agents is still increasing. The present paper deals with a preliminary experiment on Syrian hamsters into inhibition of experimental caries with glycerophosphate. The caries-reducing effect of glycerophosphate was about the same as that of sodium fluoride.

The effect of ingestion of fluorides in food and water on the incidence of fissure caries in rats

J. L. Hardwick and D. Bunting, Turner Dental School, Manchester, England

A large series of experiments has been undertaken to determine the effects of fluoride supplements to food or water on the incidence of caries in rats. Two caries

Da Costa-'44
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PHARMACOCHEMICAL STUDY OF CALCIUM GLYCEROPHOSPHATE

By Alvaro Noronha da Costa,
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Raul Leite Laboratories, S. A.

INTRODUCTION

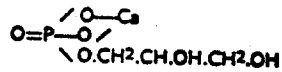
The studies of Bulow, Robin, and de Pasquali (1) demonstrate that glycerophosphoric acid and the glycerophosphates in general constitute highly esteemed and assimilable nerve tonics. Glycerophosphoric acid is one of the components of lecithin, and perhaps of some nucleoalbumins. Marfori (2) and other experimenters have proven that synthetic glycerophosphoric acid (optically inactive), used in the form of salts of calcium and of sodium, is absorbed by the digestive tract; in fact, in animals as well as men, after the swallowing of glycerophosphates, a quantity of phosphorus is found that is much less than the amount administered, according to the observations of Marfori, Tunnicliffe, and Sanson (2). The possibility of the organism's synthesizing such compounds was thoroughly studied by Mischel.(2).

The glycerophosphates, when injected under the skin, are almost entirely eliminated in the urine; this happens because the synthetic glycerophosphoric acid is not in itself assimilable, having to undergo the action of the pancreatic juices, which split the racemic compound into the two optical antepodes, dextrorotary and levorotary, the latter of which has the property of being assimilated and of serving as phosphorated nutrition for the organism, according to Marfori's studies (2). These studies were later confirmed by Maestri and Castaldi in 1915. Glycerophosphates are recommended in all cases where we need to raise the level of organic phosphorus.

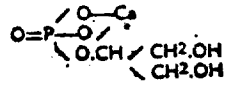
COMPOSITION OF OFFICINAL CALCIUM GLYCEROPHOSPHATE

In general, we have the impression that calcium glycerophosphate is composed merely of the mixture of two isomeric monoesters, whose formulas can be diagrammed thusly:

calcium α-glycerophosphate



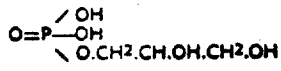
calcium β-glycerophosphate



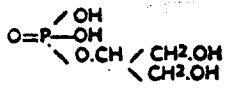
Bailly and Gaume (3), in the introduction to their study on calcium glycerophosphate, summarize the isomerism of glycerophosphoric esters in a convenient and easily understood way. This subject was also discussed by Bailly himself in his doctoral thesis for the physical sciences.

The glycerophosphoric esters are classified in three categories, namely:

Category I comprises the glycerophosphoric monoesters. The theory provides for the formation of two isomeric monoesters, corresponding to the structural formulas indicated below:



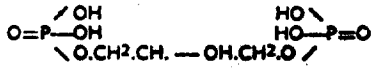
-glycerophosphoric acid



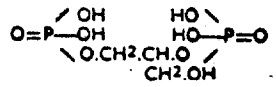
-βglycerophosphoric acid

These monoesters are remarkably stable, and their hydrolysis can be brought about only with great difficulty.

Category II comprises the glycero-diphosphoric diesters. Here again, the theory calls for two isomeric diesters, as represented by the following formulas:



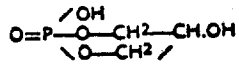
glycero- αα'- -diphosphoric acid



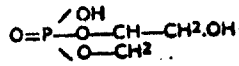
glycero- $_{\alpha\beta}$- -diphosphoric acid

Category III comprises the glycero-monophosphoric diesters. The theory provides for five diesters, and, for convenience of classification and consequent ease of comprehension, we shall divide them into two sub-categories:

Sub-category A comprises the mono-glycero-monophosphoric diesters, which are two in number, represented by the following formulas:

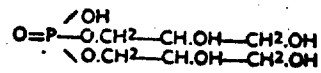


$_{\alpha\alpha'}$- -mono-glycero-monophosphoric acid

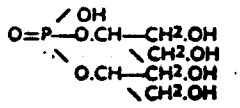


$_{\alpha\beta}$- -monoglycero-monophosphoric acid

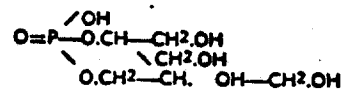
Sub-category B comprises the diglycero-monophosphoric diesters, which are three in number, and are represented by the following diagrammatic formulas:



$_{\alpha\alpha'}$- -diglyceromonophosphoric acid



$_{\beta\beta'}$- -diglycero-monophosphoric acid



αβ-diglyceromonophosphoric acid

The five glyceromonophosphoric diesters are relatively stable bodies. In an easy way (action of soda in proper theoretical proportions, diluted even when cold), we can transform them into corresponding monoesters in such a way as to have one monoglycero-monophosphoric diester or one diglycero-monophosphoric diester, according to the equations diagrammed below:

$$PO^4HC^3H^5.OH + H^2O = PO^4H^2C^3H^5(OH)^2$$

$$PO^4C^3H^5(OH)^2 + H^2O = PO^4H^2C^3H^5(OH)^2 + C^3H^5(OH)^3$$

THE COMPOSITION OF OFFICINAL CALCIUM GLYCEROPHOSPHATE AND ITS IMPORTANCE FROM THE ANALYTICAL POINT OF VIEW

We have just seen in some detail the complex structure of calcium glycerophosphate, from which we can confirm that calcium glycerophosphate is a mixture of monoesters and of diesters in variable proportions. To the analyst, this knowledge has the aspect of great importance, since on the basis of it he will not be content merely with a dosage of the monoesters, but will prescribe the diesters as well.

Some authors have raised quite an uproar against Astruc's volumetric method of prescription (3), insisting that it is not satisfactory <u>when the calcium glycerophosphate is adulterated</u>. In our opinion, this is precisely why Astruc's volumetric process should be adopted; for if the process in question does not show satisfactory results when the product under examination is adulterated, it seems logical that we should not reject it merely for this reason. On the contrary, we ought to use it, for if in a given given treatment this process is not satisfactory, we can conclude that the product under examination has been found to be adulterated, and it will consequently be rejected by any pharmacological standards whatever.

The weighing process is fraught with great difficulties, and frequently, according to what Bailly and Gaume tell us (3), is the cause of errors.

The great Rodolfo Albino wrote glowingly in our Pharmaceutical Code (7) about Astruc's volumetric process; we need only add the necessity of including in our Pharma-Standards (7) the prescription of the diesters as well.

In the chapter refering to the importance of calcium glycerophosphate from the therapeutic point of view, we demonstrate why we utilize the diesters to advantage.

In carrying out the analysis of calcium glycerophosphate, we should keep in mind the pattern established by us, which we outline below:

A. Qualitative tests.
B. Dosage of water.
C. Dosage of monoesters.
D. Dosage of hydrolyzable diesters.
E. Dosage of non-hydrolyzable diesters.

QUALITATIVE TESTS

Under this sub-title, we include only the tests of vital importance, and which our Pharmacological Standards do not record.

In the first place, let us look at the limits of acidity and of alkalinity, which are not to be found included in our Pharmaceutical Code (7).

Lebeau and Courtois (4) cite the limits prescribed by the French Pharmacological Standard, which could well be adopted by the review commision for the Brazilian Pharmacological Standards.

We translate the corresponding section, because it could be useful to that learned commision:

"Limits of alkalinity or of acidity: To the aqueous solution of 1% is added two mimims of phenolphthalein. The solution can become pink or remain colorless. In the first case, the coloration should disappear with less than two cc's of hydrochloric acid, N/10, which corresponds to an alkalinity of the product in OHNa of 0.8 grams per 100. In the second case, add a solution of sodium hydroxate N/10, until the red coloration of the phenolthalein appears; the volume of the ALKALINE solution should not be over 0.5 cc's of the said solution, which is equivalent to an acidity of the product in glycerophosphoric acid of 0.43 per cent."

We remind the office of Medical Supervision of the urgent necessity for eliminating from our market the so-called 50% solutions of calcium glycerophosphate.

We outline below, by item, the reasons favoring our position for the prohibition of the sale of the so-called 50% solutions of calcium glycerophosphate.

1. There is no solvent known at the present time that is capable of dissolving calcium glycerophosphate in such a proportion.

2. Logically, from the previous item, we infer the impossibility of our obtaining a solution of calcium glycerophosphate of 50%.

3. In such solutions, the diesters are eliminated by filtration.

4. These solutions are greatly subject to fraudulency.

One of these frauds which came into our hands was sent by our colleague Ary de Almeida Rios. Our good friend noticed some sort of abnormality in the mentioned solution, and, knowing that we were conducting a study about calcium glycerophosphate, decided to send us, on pretense of curiosity, a sample of that solution. Our colleague was astonished to learn that the solution of calcium glycerophosphate was nothing more than a glycero-aqueous solution of calcium phosphate.

5. Solutions of calcium glycerophosphate show a very high level of acidity, and in some, besides citric acid, we find phosphoric acid, which is not permitted by our Pharmacological Standards in natural salt.

Bringing to a close our considerations about the so-called 50% solutions of calcium glycerophosphate, we beg leave to transcribe a table which we found in the work of pharmacist E. Delvaux (6) of the University of Louvain, entitled "Analysis of Calcium Glycerophosphate."

TABLE ORGANIZED BY E. DELVAUX (6)

	Moisture at 130°	Calcium	P_2O_5	Monoester
I. Calcium Glycero-		18.66 g.%	33.80 g.%	99.80 g.%
phosphate Ph. B. IV	12.10 g.%	18.59 g.%	33.85 g.%	98.92 g.%
II. Soluble Glycero-		16.73 g.%	29.45 g.%	
phosphate (acid to phenolphthalein)	14.40 g.%	16.73 g.%	29.38 g.%	
III Calcium Glycero-		3.90 g.%	16.30 g.%	
phosphate called 50%		3.93 g.%	16.45 g.%	

INTERPRETATION OF TABLE

The table is of interest to us solely for the outcomes of Samples I and III. Sample I is formed exclusively of monoesters, as we can perceive by comparing the percentage of calcium-phosphorus obtained by the said author with the percentage of calcium-phosphorus which a pure glycerophosphate made up of only monoesters would have.

SAMPLE I:
Outcome: Calcium %: 18.6 grams
Phosphorus %: 14.7 grams or 33.8 in P_2O_5

GLYCEROPHOSPHATE MADE UP SOLELY OF MONOESTERS: THEORETICAL PROPORTION OF PHOSPHORUS AND CALCIUM

Calcium %: 19 grams
Phosphorus %: 14.7 grams

What we have claimed, by the way, is the simple truth, since in the table referred to, the author included in Sample I the respective monoesters; according to the recorded outcome: that is, 99% of monoesters.

OBSERVATIONS: The table in question shows the outcome in phosphoric oxide, and we have calculated in terms of phosphorus to facilitate understanding.

The outcome of Sample III represents the content of phosphorus and of calcium in one of the so-called 50% solutions of calcium glycerophosphate. If Sample III were a 50% solution, the percentages of phosphorus and of cal-

cium ought to be half, or approximately half, of the percentages of phosphorus and calcium in Sample I. However, we do not observe this, but rather we see a reduction in the level of calcium and a quantity quite close to normal in the percentage of phosphorus, as is shown in the outline below:

SAMPLE I IN 50 GRAMS OF THE PRODUCT:

Calcium: 9.3 grams
Phosphorus: 7.35 grams, or 33.8 in P_2O_5

SAMPLE III IN 100 GRAMS OF THE PRODUCT:

Calcium: 3.93 grams
Phosphorus: 7.16 grams, or 16.4 grams of P_2O_5

CONCLUSIONS

(a) Sample III, said to be a 50% solution, does not contain 50% of calcium glycerophosphate.

(b) The difference in reported phosphorus and calcium leads us to conclude that the 50% solution was adulterated with phosphoric acid, hence the increased level of phosphorus.

(c) The effect exerted by phosphate acids in organic decalcification is well known today.

(d) We should, moreover, put an end to the so-called glycerophosphate solutions, for theirbeing socially harmful.

Further under the heading of qualitative tests, we call the attention of the Brazilian Pharmacological Standards review commission to the research on citric acid, which our Pharmacological Standard has not codified, but on which there is need for further research because it is one of the most common frauds. Citric acid is added for the purpose of increasing the solubility of calcium glycerophosphate.

Lebeau and Courtois (4) say that citric acid profoundly modifies the nature of the salt. In the Dictionary of Medicaments (1) we find a noteregarding the use of citric acid to increase the solubility of calcium glycerophosphate in syrupy liquids; it is an interesting reminder that this note shows us the formation of calcium citrate by double exchange.

We present below a technique for research on citric acid which perhaps could be useful to the review commission of the Brazilian Pharmacological Standards (7).

Technique: Dilute 1 gram of powdered calcium glycerophosphate with 25 cc's of pure alcohol, filter, and evaporate the filtrate. Dissolve the residue in distilled water, put into a test tube, add one cc. of Deniges reagent, and bring to a boil. Add a few drops of a N/10 solution of potassium permanganate. In the positive case, we obtain a white opalescence, or white pp. (potassium permanganate?).

MOISTURE CONTENT

The details of the technique will not be explained; we simply recall the routine which we follow: We put two grams of the product into a capsule of known weight, and heat to 150° C. We dry to a constant weight, weigh again, and calculate the percentage of moisture.

Observations: Some authors feel that it should become dry at a temperature of 130° C. Bailly and Gaume (3), who studied the subject quite thoroughly, say that only at 150° can the moisture be eliminated from calcium glycerophosphate. We call attention to an interesting point in the studies of these two authors, regarding the question of the partial hydrolysis of the diesters at a temperature of 150° C.

This observation is valid, since it is for this reason that we do not use the same step in the experiment to test for moisture content and also for monoester and diester content.

At first glance, it seems to us that a cause for error could arise, but this does not take place because the products of this hydrolysis are fixed at a temperature of 150°C.

MONOESTER CONTENT

Technique: We weigh 1 gram of the product, dissolve it in 100 cc's of distilled water, add a minim of phenolphthalein and two minims of helianthin. We treat the filtered phenolphthalein-helieanthin mixture with an N/2 solution of hydrochloric acid. We record the number, "N", of cc's of this solution.

HYDROLYZABLE DIESTER CONTENT

Technique: We weigh 1 gram of the product, dissolve it in 100 cc's of distilled water, add 10 cc's of an N/2 solution of sodium hydroxate, and heat for half an hour. We add a minim of phenolphthalein and two minims of helianthin, after cooling. We treat the filtered phenolphthalein-helianthin with an N/2 solution of hydrochloric

in monoesters) . . . . . 5.25 g.
Total . . . . . 85 g.
Percentage of non-hydrolyzable diesters: 100 - 85 = 15 g.

As the product being examined proves positive only to the reaction of chlorides, for greater precision in the amount of non-hydrolyzable diesters, we treat with chlorides, and we find, expressed in calcium chloride, 1.2 g. %.

Thus the percentage of non-hydrolyzable diesters = 15 - 1.2 = 13.8 g. %.

We show in the following table the summary of analyses conducted.

SUMMARY TABLE

Sample Number	Moisture at 150°	Mono-esters	Hydrolyzable diesters	Non-hydrolyzable diesters
I	10.5 g.%	87.21 g%	1.57 g.%	0.72 g.%
II	10.2 g.%	86.1 g.%	2.1 g. %	1.6 g.%
III	11.5 g.%	86.1 g.%	none	2.3 g.%
IV	10.24 g%	89.25 g%	none	0.51 g.%
V	12.0 g.%	67.75 g%	5.25 g.%	13.8 g.%

THE COMPOSITION OF CALCIUM GLYCEROPHOSPHATE AND ITS IMPORTANCE FROM THE THERAPEUTIC POINT OF VIEW

We return once again to discuss the outcome of Sample V, since it really constitutes the purpose of our study. Our Pharmacological Standard disapproves of this glycerophosphate because of its low monoester content. After testing the glycerophosphate of Sample V, we found it to be qualitatively perfect. The positive reaction of chlorides could not call it into question, because the Brazilian Pharmacological Standards (7) permit a small quantity of chlorides. For better guidance, we measured the chlorides, and found that the quantity of chlorides is not great.

We were facing a problem whose solution at first presented great difficulty. We began a study of the subject, linking the existing literature to practical experiments. It was the combination of both that led us to write this study, whose only purpose is to standardize what is efficacious in our pharmaceutical market and reject the useless.

We find that, after the complete outcome of the analysis of Sample V, this glycerophosphate of calcium can be employed for pharmaceutical use.

Our contention is based upon a hypothesis that can be confirmed or disputed only by pharmacologists. In the introduction to our study we referred to the studies of Marfori (2), later confirmed by Maestri and Castaldi, regarding the action of pancreatic juices upon synthetic glycerophosphoric acid. We submit the hypothesis that the glycero-diphosphoric esters undergo the action of the pancreatic juice, being transformed into the respective monoesters. Should our hypothesis be correct, we launch in defense of Sample V a pattern of percentages of phosphorus in the various glycerophosphoric esters.

Thus, we have:

A) Glycerophosphoric monoesters . . . . . 15 g
B) Glycero-diphosphoric diesters . . . . . 19 g
C) Glycero-monophosphoric diesters:
  1) Monoglycero-monophosphoric diesters . . . 18 g
  2) Diglycero-monophosphoric diesters . . . 12 g

<u>Observations</u>: The percentage of phosphorus which we show above is the theoretical quantity of phosphorus corresponding to gram molecule of the ester. For ease of comparison, we decided to give the results in whole numbers, rounding out those which went over a half.

A comparative study of the percentage of phosphorus in the various glycerophosphoric esters shows us that the glycero-diphosphoric esters have a higher percentage of phosphorus than do the other esters. Since, as we have already said, glycerophosphates are recommended in all cases where there is a need for us to increase the level of organic phosphorus, the reason for our support of Sample V is readily inferred.

We observed that commercial calcium glycerophosphates are not soluble in the proportions of water generally established in the text-books. This observation, incidentally, is not ours, but rather that of Bailly and Gaume; we merely confirm its validity.

The solubility of calcium glycerophosphate can be influenced either by the presence of calcium phosphate, in which case the product should be rejected, or by the presence of glycero-diphosphoric diesters, whose calcium salts are not very soluble in water, in which case the product can be utilized if we observe the standard which we shall establish below. Before presenting the standard for the use of glycerophosphate which contains glycero-diphosphoric esters, we are going to show how we can make the

differentiation between these esters and calcium phosphate, when present in a glycerophosphate.

We take one gram of the product, dissolve it in 40 cc's of distilled water, and filter; we treat the residue which remains upon the filter with water acified by nitric acid, and test for phosphate in the solution, with the reagent molybdic nitrate; if the reaction is positive, a phosphate of calcium is present; if negative, a diester is present.

The difficulty of our establishing exactly the solubility of officinal calcium glycerophosphate (consisting of a mixture of mono- and di-esters in variable proportions) leads us to propose a standard for its therapeutic use. This standard is nothing more than the connecting together of deductions which seem logical to us. Contrary opinions, however, could be presented.

The standard itself seeks to prevent the glycero-diphosphoric diesters, insufficiently soluble in water, from being retained by the filtration which normally is done in the pharmacies.

"Thus, we propose that calcium glycerophosphate be prescribed in a gummy potion."

This standard involves a secondary factor, which if not clarified could become a problem. For us to know what this factor is, we must add to our preceding proposition an assertion: "The gummy potion should be made in an inhibiting (adragante) gum, or other gum that does not contain peroxydasic or oxydasic enzymes.

Let us explain the reason for this assertion: J. Rae (5), studying the successive action of sulphuric acid, soda, glucose, and sugar cane upon glycerophosphates, concluded, after colorimetric treatment of the phosphate, that soda and glucose did not cause detachment of glycerophosphate experimentally; sulphuric acid and sugar cane produced in six months a detachment corresponding respectively to 0.5% and 0.6% of phosphate. Continuing his studies, he found that the peroxydases of gum arabic were incompatible with the glycerophosphates, transforming them partially into phosphates.

CONCLUSIONS

(1) We wish to emphasize the studies of Marfori, Maestro, and Castaldi in respect to the non-absorption of synthetic glycerophosphoric acid, when assimilated otherwise than through the alimentary tract.

(2) Astruc's volumetric process, combined with qualitative tests, permits to exercise satisfactory control over officinal calcium glycerophosphate.

(3) Among the qualitative tests, we cannot omit the limits of alkalinity and of acidity, as well as the investigation of citric acid.

(4) The procedure which we established for control of calcium glycerophosphate enables us to determine with greater precision whether the product will or will not satisfy the requisite demands.

(5) The evidence which we have presented against the so-called 50% solutions of calcium glycerophosphate are sufficient for the competent authorities to take preventive measures regarding them.

(6) We must extend in our pharmaceutical code the limit of solubility of officinal calcium glycerophosphate.

(7) Pharmacologists should test experimentally whether or not the glycero-diphosphoric diesters are absorbed by the body.

(8) Should it be proven that pancreatic juices cannot successfully separate these diesters, it will be necessary to establish a limit on them in officinal calcium glycerophosphate.

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Estudo Fármaco-Químico do Glicerofosfato de Cálcio

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Da Secção de Análise e Pesquisas dos Laboratórios Raul Leite S. A.

"...Êste trabalho, cujo único fito é padronizar o que há de oficinal..."

INTRODUÇÃO

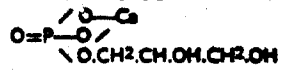
OS estudos de Bulow, Robin e de Pasquali (1) demonstraram que o ácido glicerofosfórico e os glicerofosfatos em geral constituem apreciadíssimos tônicos nervosos, assimiláveis. O ácido glicerofosfórico é um dos componentes da lecitina e talvez de algumas núcleo-albuminas. Marfori (2) e outros experimentadores provaram que o ácido glicerofosfórico sintético (inativo òticamente) usado sob a forma de sais de cálcio ou de sódio é absorvido pelo tubo digestivo; de fato, tanto nos animais como no homem após a ingestão de glicerofosfatos, encontra-se na urina e nas fezes uma quantidade de fósforo muito menor do que a administrada, conforme observaram Marfori, Tunnicliffe e Sanson. (2) A possibilidade de o organismo sintetisar tais compostos foi muito bem estudada por Mischel (2).

Os glicerofosfatos, quando injetados sob a pele, são quase totalmente eliminados com a urina; isto se dá porquê o ácido glicerofosfórico sintético não é por si só assimilável, havendo necessidade de sofrer a ação do suco pancreático, que cinde o composto racêmico nos dois antípodes óticos, dextrógiro e levógiro, dos quais o último tem propriedade de ser assimilado e de servir à nutrição fosforada do organismo segundo os estudos de Marfori (2). Êsses estudos foram depois confirmados por Maestro e Castaldi em 1915. Os glicerofosfatos são indicados em todos casos em que há necessidade de elevarmos a taxa de fósforo orgânico.

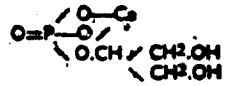
COMPOSIÇÃO DO GLICEROFOSFATO DE CÁLCIO OFICINAL:

Temos em geral a impressão de que o glicerofosfato de cálcio é constituido apenas pela mistura de 2 monoésteres isômeros, cujas fórmulas podem ser esquematizadas assim:

α-glicerofosfato de cálcio



β-glicerofosfato de cálcio



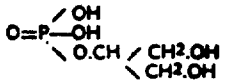
Bailly e Caumé (3) resumem, na introdução de seu trabalho sôbre o glicerofosfato de cálcio, a isomeria dos ésteres-glicerofosfóricos

de maneira acessível e fácil de ser compreendida. Aliás êsse assunto foi ventilado pelo próprio Bailly em sua tese para doutorado em ciências físicas.

Os ésteres glicerofosfóricos são classificados em 3 categorias, a saber:

1.ª Categoria compreende os monoésteres glicerofosfóricos. A teoria prevê a formação de 2 monoésteres isômeros, respondendo pelas fórmulas estruturais abaixo indicadas:

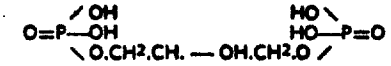
ácido α-glicerofosfórico



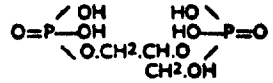
ácido β-glicerofosfórico

Esses monoésteres são ésteres notàvelmente estáveis e sua hidrólise só se efetua por meios enérgicos.

2.ª Categoria compreende os diésteres glicero-difosfóricos. A teoria prevê igualmente 2 diésteres isômeros, respondendo pelas seguintes fórmulas:



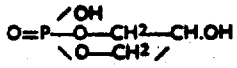
ácido glicero $\alpha\alpha'$-difosfórico



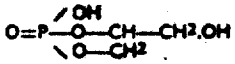
ácido glicero $\alpha\beta$-difosfórico

3.ª Categoria compreende os diésteres glicero-monofosfóricos. A teoria prevê 5 diésteres e, por comodidade de classificação e conseqüente facilidade de compreensão, dividiremos em duas sub-categorias:

Sub-categoria A compreende os diésteres mono-glicero-monofosfóricos que são em número de 2, respondendo pelas seguintes fórmulas:

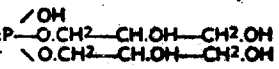


ácido $\alpha\alpha'$-monoglicero-mono-fosfórico

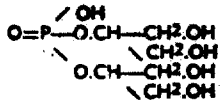


ácido $\alpha\beta$-monoglicero-monofosfórico

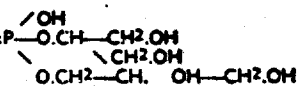
Sub-categoria B compreende os diésteres-diglicerómono-fosfóricos que são em número de 3 e apresentam esquemàticamente as seguintes fórmulas:



ácido $\alpha\alpha'$-digliceromonofosfórico

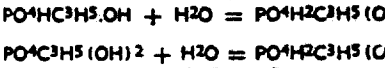


ácido $\beta\beta'$-diglicero-monofosfórico

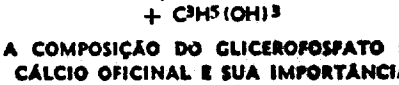


ácido $\alpha\beta$-digliceromonofosfórico

Os cincos diésteres gliceromonofosfóricos são corpos relativamente instáveis. Por meio suave (ação da soda em proporção teórica, diluida mesmo a frio) podemos transformá-los em monoésteres correspondentes conforme tenhamos um di-éster — monoglicero-monofosfórico ou um di-éster-digliceromono-fosfórico, segundo as equações abaixo esquematizadas:



$$PO^4HC^3H^5.OH + H^2O = PO^4H^2C^3H^5(OH)^2$$



$$PO^4C^3H^5(OH)^2 + H^2O = PO^4H^2C^3H^5(OH)^2 + C^3H^5(OH)^3$$

A COMPOSIÇÃO DO GLICEROFOSFATO DE CÁLCIO OFICINAL E SUA IMPORTÂNCIA SOB O PONTO DE VISTA ANALÍTICO:

Vimos anteriormente com alguns detalhes a complexa estrutura do glicerofosfato de cálcio e daí podermos afirmar que o glicerofosfato de cálcio é uma mistura de monoésteres e de diésteres em proporções variáveis. Para um analista êsses conhecimentos revestem-se de grande importância, pois munido dos mesmos não se contentará apenas com o dosearmento dos monoésteres e sim doseará também os diésteres.

E' grande a celeuma feita por alguns autores contra o processo de doseamento volumétrico de Astruc (3), afirmando que o mesmo não satisfaz quando o **glicerofosfato de cálcio está falsificado.** Eis em a nossa opinião por que deva ser adotado o processo volumétrico de Astruc (3); pois se o processo em questão, só não apresenta resultados satisfatórios quando o produto em exame está falsificado, é lógico que não devemos desprezá-lo por isso. Pelo contrário, devemos utilizá-lo por que se em um doseamento o referido processo não satisfizer, podemos afirmar que o produto em exame se acha falsificado e portanto será impugnado por qualquer Farmacopéia.

O processo ponderal reveste-se de grandes dificuldades técnicas e não raras vezes, conforme nos afirma Bailly e Caumé (3), apresenta causas de erro.

O grande Rodolfo Albino inscreveu em o nosso código farmacêutico (7) com muita felicidade o processo volumétrico de Astruc; temos que acrescentar apenas a necessidade de incluirmos em a nossa Farmacopéia (7) o doseamento dos diésteres também.

No capítulo referente a importância do glicerofosfato de cálcio sob o ponto de vista terapêutico, demonstramos porquê fizemos o aproveitamento dos diésteres.

Ao efetuarmos a análise de um glicerofosfato de cálcio devemos ter em mente o paradigma por nós estabelecido, que esquematizamos abaixo:

A - Ensaios qualitativos.
B - Doseamento da umidade (água).
C - " dos monoésteres.
D - " dos diésteres hidrolisáveis.
E - " dos diésteres não hidrolisáveis.

ENSAIOS QUALITATIVOS

Com êsse sub-título, abrangemos apenas os ensaios de importância capital e que a nossa Farmacopéia não consigna.

Em primeiro lugar vejamos os limites de acidez e de alcalinidade que não se acham inseridos em o nosso código farmacêutico (7).

Lebeau e Courtois (4) citam os limites prescritos pela Farmacopéia Francesa, que bem poderiam ser adotados pela comissão revisora da Farmacopéia Brasileira.

Traduzimos o trecho correspondente porquê poderá ser útil àquela douta comissão:

"Limites de alcalinidade ou de acidez — À solução aquosa a 1% juntam-se 2 gotas de fenolftaleina. A solução pode se colorir de rosea ou ficar incolor. No primeiro caso, a coloração deve desaparecer com menos de 2 cm³ de ácido clorhídrico $\frac{N}{10}$, que corresponde a uma alcalinidade do produto em OHNa de 0,8 g por cento. No segundo caso, junta-se solução de oxhidrato de sódio $\frac{N}{10}$ até aparecer a coloração rósea da fenolftaleina; o volume de solução ALCALINA não deve ser superior a 0,5 cm³ de sol. titulada, que equivale a uma acidez do produto em ácido glicerofosfórico de 0,43 por cento".

Lembramos ao serviço de Fiscalização da Medicina a necessidade imperiosa de eliminar do nosso comércio as chamadas soluções de glicerofosfato de cálcio a 50%.

Esquematizamos mais adiante, em itens, os porquês da nossa tese favorável à proibição de venda das chamadas soluções a 50% de glicerofosfato de cálcio.

1.º) Não há solvente conhecido até a data de hoje, que seja capaz de dissolver o glicerofosfato de cálcio em tal proporção.

2.º) Logicamente do item anterior, inferimos a impossibilidade de obtermos solução de glicerofosfato de cálcio na proporção de 50%.

3.º) Em tais soluções, os diésteres são eliminados por filtração.

4.º) Essas soluções estão muito sujeitas a fraudes.

Uma dessas fraudes veio ter às nossas mãos enviada pelo colega Ary de Almeida Rios. O ilustre amigo percebeu qualquer anormalidade em a referida solução e sabendo que nós estávamos elaborando um trabalho sôbre glicerofosfato de cálcio, resolveu [illegible] de curiosidade uma amostra [illegible]. Pasmado ficou o nosso confrade quando soube que a solução de glicerofosfato de cálcio nada mais era do que uma solução [illegible] aquosa de fosfato de cálcio.

5.º) As soluções de glicerofosfato de cálcio apresentam uma acidez muito grande e em algumas além do ácido cítrico encontramos o ácido fosfórico que não é permitido pela nossa Farmacopéia no sal em natureza.

Terminando nossas considerações sôbre as chamadas soluções a 50% de glicerofosfato de cálcio, pedimos vênia para transcrever um quadro que encontramos em o trabalho do farmacêutico E. Delvaux (6) da Universidade de Louvain, intitulado "Análise do glicerofosfato de cálcio".

QUADRO ORGANIZADO POR E. DELVAUX (6)

	Umidade a 130º	Cálcio	P_2O_5	Monoéster
I — Glicerofosfato de Cálcio Ph. B. IV . . .	12.10 g. %	18.66 g. % 18.59 g. %	33.80 g. % 33.85 g. %	99.80 g. % 98.92 g. %
II — Glicerofosfato Solúvel (ácido à Fenolftaleina)	14.40 g. %	16.73 g. % 16.73 g. %	29.45 g. % 29.38 g. %	
III — Glicerofosfato de Cálcio . . . . . . Líquido dito a 50%		3.9 g. % 3.93 g. %	16.30 g. % 16.45 g. %	

INTERPRETAÇÃO DO QUADRO

O quadro apresenta importância para nós apenas pelos resultados das amostras I e III. A amostra I é exclusivamente formada de monoésteres como podemos observar comparando a percentagem fósforo-cálcio obtida pelo referido autor com a percentagem fósforo-cálcio que tem teoricamente um glicerofosfato puro constituido apenas de monoésteres.

AMOSTRA N.º 1

Resultado: Cálcio %: 18,6 g.
Fósforo %: 14,7 g ou 33,8 em P_2O_5

GLICEROFOSFATO CONSTITUIDO APENAS DE MONOÉSTERES

PROPORÇÃO TEÓRICA DE FÓSFORO E CÁLCIO

Cálcio %: 19 g.
Fósforo %: 14.7 g.

Aliás o que afirmamos é a pura verdade, pois no referido quadro o autor doseou em a amostra número 1 os respectivos monoésteres, conforme o resultado catalogado, isto é 99% de monoésteres.

OBSERVAÇÕES: O quadro em questão apresenta o resultado em óxido fosfórico e nós calculamos em fósforo para facilitar a compreensão.

O resultado da amostra número III representa o doseamento do fósforo e do cálcio em uma das chamadas soluções a 50% de glicerofosfato de cálcio. Sendo a amostra III uma solução a 50%, a percentagem de fósforo e do cálcio devia ser a metade da percentagem fosfóro-cálcio da amostra I ou aproximadamente a metade. Entretanto não é isso que observamos, mas sim uma diminuição da taxa de cálcio e uma quantidade bem próxima da normal em a percentagem de fósforo, conforme mostra o esquema que reproduzimos adiante:

AMOSTRA I EM 50 g. DO PRODUTO
Cálcio — 9,3 g.
Fósforo — 7,35 g. ou 33,8 em P_2O_5

AMOSTRA III EM 100 g. DO PRODUTO
Cálcio — 3,93 g.
Fósforo — 7,16 g. ou 16,4 g. em P_2O_5

CONCLUSÕES

a) A amostra número III dita solução a 50% não contém 50% de glicerofosfato de cálcio.

b) O disparate da relação fósforo-cálcio leva-nos à conclusão de que a solução a 50% foi adicionada de ácido fosfórico, daí o aumento da taxa de fósforo.

c) Hoje em dia, já está esclarecida a influência que exercem os fosfatos ácidos na descalcificação orgânica.

d) Devemos, pois, acabar com as chamadas soluções de glicerofosfato de cálcio por serem prejudiciais à coletividade.

Ainda sob o título de ensaios qualitativos chamamos a atenção da comissão revisora da Farmacopéia Brasileira para a pesquisa do ácido cítrico, que a nossa Farmacopéia não codificou, mas há necessidade de pesquisá-lo por ser uma das fraudes mais comuns. Adicionam o ácido cítrico com o fim de aumentar a solubilidade do glicerofosfato de cálcio.

Lebeau e Courtois (4) dizem que o ácido cítrico modifica profundamente a constituição do sal. Em o dicionário de Medicamentos (1) encontramos uma nota sôbre o emprêgo do ácido cítrico para aumentar a solubilidade do glicerofosfato de cálcio nos líquidos xaroposos; é interessante lembrar que esta nota nos mostra a formação do citrato de cálcio por dupla troca.

Damos a seguir uma técnica para a pesquisa do ácido cítrico que talvez possa ser aproveitada pela comissão revisora da Farmacopéia Brasileira (7).

Técnica: "Triture 1 g de glicerofosfato de cálcio com 25 cm³ de alcool absoluto, filtre e evapore o filtrado. Dissolva o resíduo em água destilada, passe para um tubo de ensaio, junte 1 cm³ do reativo de Denigès e leve à ebulição. Trate em seguida por algumas gotas de solução N/10 de permanganato de potássio. Em caso positivo obtemos uma opalescência branca ou pp. branco".

DOSEAMENTO DA UMIDADE

Os detalhes de técnica não serão explanados, lembramos apenas a marcha que adotamos: "Pesamos 2 g do produto em cápsula tarada e levamos à estufa a 150°C. Dessecamos até pêso constante, pesamos novamente e calculamos a percentagem de umidade".

Observações: Alguns autores acham que se deve dessecar a temperatura de 130°C. Bailly e Gaumé (3) que estudaram muito bem o assunto, dizem que só a 150°C se consegue eliminar a umidade do glicerofosfato de cálcio. Chamamos a atenção para um ponto interessante em os estudos dos dois autores anteriormente citados, é sôbre a questão da hidrólise parcial dos diésteres na temperatura de 150°C.

Essa observação é valiosa, pois assim não utilizaremos a mesma tomada de ensaio para doseamento da umidade e dos monoésteres ou dos diésteres.

Parece-nos à primeira vista que uma causa de erro pode se dar, no entanto isso não acontece porquê os produtos dessa hidrólise são fixos à temperatura de 150°C.

DOSEAMENTO DOS MONOÉSTERES

Técnica: Pesamos 1 g do produto, dissolvemos em 100 cm³ de água destilada, juntamos uma gota de fenolftaleina e duas gotas de heliantina. Doseamos a passagem fenolftaleina-heliantina com solução N/2 de ácido clorhídrico. Anotamos o número "N" de cm³ de solução titulada.

DOSEAMENTO DOS DIÉSTERES HIDROLISÁVEIS

Técnica: Pesamos 1 g do produto, dissolvemos em 100 cm³ de água destilada, adicionamos 10 cm³ de solução N/2 de oxhidrato de sódio e aquecemos durante 1/2 hora em B.M. Juntamos uma gota da fenolftaleina e duas gotas de heliantina, após resfriamento. Doseamos a passagem fenolftaleina-heliantina com solução N/2 de ácido clorhídrico. Anotamos o número de cm³ de solução titulada "n".

CÁLCULO DOS MONOÉSTERES E DOS DIÉSTERES HIDROLISÁVEIS

O teor de monoésteres é calculado com a seguinte fórmula:

Monoésteres % = NxO, 1050765×100.

O teor dos diésteres é calculado com a seguinte fórmula:

Diésteres % = (n-10-N) 0,1050765×100.

Observações: Quando explanamos anteriormente a questão da isomeria dos diésteres glicerofosfóricos, vimos que os diésteres-glicero-monofosfóricos foram divididos em duas sub-categorias:

Sub-categoria A — Compreendendo os diésteres-monoglicero-monofosfóricos.

Sub-categoria B — Compreendendo os diésteres-diglicero-monofosfóricos.

Essas duas sub-categorias apresentam diésteres com pêsos moleculares diferentes. Afim de padronizarmos os resultados obtidos e conseguirmos ultrapassar a dificuldade exposta, resolvemos calcular o resultado dos diésteres em monoésteres. Sabemos pelo esquema de equações já apresentado nesse trabalho, que podemos obter uma transformação quantitativa dos diésteres em monoésteres correspondentes, sob a ação da soda.

1) Diésteres-monoglicero-monofosfóricos 18g
2) Diésteres diglicero-monofosfóricos .. 12g

Observações: A percentagem de fósforo que indicamos acima é a quantidade teórica de fósforo relacionada à molécula-grama do éster. Para facilitar a comparação, resolvemos dar os resultados em números inteiros, aproximando os que ultrapassassem de meio.

Um estudo comparativo da percentagem de fósforo nos diversos ésteres-glicero-fosfóricos mostra-nos que os diésteres glicero-difosfóricos apresentam uma percentagem de fósforo superior à dos outros ésteres. Sendo, como já dissemos, os glicerofosfatos indicados em todos os casos que há necessidade de aumentarmos a taxa de fósforo orgânico, facilmente se infere o porquê da nossa defesa pela amostra V.

Observamos que os glicerofosfatos de cálcio comerciais não se solubilizam em as proporções de água estabelecidas geralmente pelos compêndios. Aliás essa observação não é nossa, mas sim de Bailly e Coumé, nós apenas confirmamos a sua veracidade.

A solubilidade do glicerofosfato de cálcio pode ser influenciada pela presença de fosfato de cálcio, nesse caso o produto deve ser rejeitado, ou pela presença de diésteres-glicero-difosfóricos, cujos sais de cálcio são pouco solúveis na água e nesse caso o produto pode ser aproveitado se observarmos a norma que traçamos mais adiante. Antes de apresentarmos a norma para emprêgo do glicerofosfato que contém diésteres-glicero-difosfóricos, vamos demonstrar como podemos fazer a diferenciação entre esses diésteres e o fosfato de cálcio, quando presentes em um glicerofosfato.

"Tomamos 1 g do produto, dissolvemos em 40 cm³ de água destilada e filtramos; tratamos o resíduo que ficou sôbre o filtro com água acidulada pelo ácido nítrico e pesquisamos fosfato nessa solução, com o reagente nitromolibídico; se a reação for positiva estamos na presença de um fosfato de cálcio, se negativa na presença de um diéster".

A dificuldade de estabelecermos exatamente a solubilidade do glicerofosfato de cálcio oficinal (constituído de uma mistura de mono e diésteres em proporções variáveis) leva-nos a propor uma norma para o seu emprêgo terapêutico. Essa norma nada mais é do que a concatenização de deduções que nos parecem lógicas. Pode porém apresentar opiniões contraditórias.

A norma em si visa evitar que os diésteres-glicero-difosfóricos, pouco solúveis em água, fiquem retidos pela filtração que normalmente se efetua nas Farmácias.

"Assim propomos que o glicero-fosfato de cálcio seja receitado em poção gomosa".

Essa norma prevê um fator secundário que se não fôr aclarado tornar-se-á um problema. Para sabermos qual é êsse fator, temos que juntar à nossa proposição anterior uma afirmativa: "A poção gomosa deve ser feita em goma adragante ou outra goma que não apresente enzimas: peroxidases — oxidases".

Expliquemos a razão do ser da nossa afirmativa: J. Rae (5), estudando a ação sucessiva do ácido sulfúrico, da soda, da glicose e do açúcar de cana sôbre os glicerofosfatos, concluiu após dosagem colorimétrico do fosfato que a soda e a glicose não produziram desmembramento do glicero-fosfato em experiência; o ácido sulfúrico e o açúcar de cana produziram em 6 meses um desmembramento correspondente respectivamente a 0,5% e 0,6% de fosfato. Continuando seus estudos, verificou que as peroxidases da goma arábica eram incompatíveis com os glicerofosfatos, pois transformava-os parcialmente em fosfatos.

CONCLUSÕES

1) Desejamos ressaltar os estudos de Marfort, Maestro e Cartaldi a respeito da não absorção do ácido glicerofosfórico sintético, quando introduzido por via parenteral.

2) O processo volumétrico de Astruc associado aos ensaios qualitativos permite-nos exercer um contrôle satisfatório do glicerofosfato de cálcio oficinal.

DOSEAMENTO DOS DIÉSTERES NÃO HIDROLISÁVEIS

O doseamento dos diésteres não hidrolisáveis propriamente não é doseamento mas apenas uma avaliação da ordem de grandeza obtida por diferença.

Tomemos, como exemplo, a amostra V do quadro que reproduzimos mais adiante. Após efetuarmos os ensaios prescritos pela nossa Farmacopéia (7) e mais os que citamos em o nosso trabalho, consideramos o produto em exame qualitativamente perfeito.

Assim temos como resultado da amostra V:

1) **Umidade %** ..... 12 g
2) **Monoésteres %** .....: 67,75 g
3) **Diésteres Hidrolisáveis** (calculados em monoésteres) % . 5,25 g

Total ..... 85 g

Diést. não Hidrolisáveis % (100—85 = 15 g)

Como o produto em exame apresentava positiva apenas a reação dos cloretos, para maior precisão do resultado em diésteres não hidrolisáveis, doseamos os cloretos e encontramos expresso em cloreto de cálcio 1,2 g %.

Logo os diésteres não hidrolisáveis

% = 15 — 1,2 = 13,8 g %

Damos a seguir o quadro esquemático das análises efetuadas.

QUADRO ESQUEMÁTICO

Amostras	Umidade a 150°	Monoésteres	Diést. Hidrosilíveis	Diést. não Hidrolisáveis
I	10,5 g %	87,21 g %	1,57 g %	0,72 g %
II	10,2 g %	86,1 g %	2,1 g %	1,6 g %
III	11,5 g %	86,1 g %	não tem	2,35 g %
IV	10,24 g %	89,25 g %	não tem	0,51 g %
V	12,0 g %	67,75 g %	5,25 g %	13,8 g %

A COMPOSIÇÃO DO GLICEROFOSFATO DE CÁLCIO E SUA IMPORTÂNCIA SOB O PONTO DE VISTA TERAPÊUTICO

Voltamos mais uma vez a falar sôbre o resultado da amostra V, pois em verdade constitue a razão de ser do nosso trabalho. A nossa Farmacopéia condenou êsse glicerofosfato pelo seu baixo teor em monoésteres. Depois de ensaiarmos o glicerofosfato da amostra V, verificamos que qualitativamente êle estava perfeito. A reação de cloretos positiva não podia impugná-lo, porquê a Farmacopéia Brasileira (7) permite uma pequena quantidade de cloretos. Para melhor orientação, doseamos os cloretos e observamos que a quantidade de cloretos não é grande.

Estavámos à frente de um problema cuja solução se revestia inicialmente de grande dificuldade. Iniciamos um estudo do assunto, reunindo a bibliografia existente, os experimentos práticos. A concatenização de ambos levou-nos a escrever êsse trabalho, cujo único fito é padronizar o que há de eficiente em o nosso comércio de drogas e expurgá-lo das inutilidades. Achamos que, após o resultado completo da análise da amostra V, êsse glicerofosfato de cálcio pode ser empregado para uso farmacêutico.

A nossa afirmação está fundamentada em uma hipótese, que só os farmacologistas podem afirmar ou contestar. Na introdução do trabalho fizemos referências aos estudos de Marfori (2), confirmados depois por Maestro e Castaldi a respeito da ação do suco pancreático sôbre o ácido glicerofosfórico sintético. Emitimos a hipótese de que os diésteres-glicero-di-fosfóricos sofrem a ação do suco pancreático, transformando-se em monoésteres respectivos. Sendo a nossa hipótese verdadeira, lançamos em defesa da amostra V um esquema da percentagem de fósforo nos diversos ésteres-glicerofosfóricos.

Assim temos:

A) **Monoésteres glicerofosfóricos** .... 15 g
B) **Diésteres glicero-di-fosfóricos** .... 19 g
C) **Diésteres glicerometafosfóricos:**

1) Diésteres-monoglicero-monofosfóricos 18g
2) Diésteres diglicero-monofosfóricos .. 12g

Observações: A percentagem de fósforo que indicamos acima é a quantidade teórica de fósforo relacionada à molécula-grama do éster. Para facilitar a comparação, resolvemos dar os resultados em números inteiros, aproximando os que ultrapassassem de meio.

Um estudo comparativo da percentagem de fósforo nos diversos ésteres-glicero-fosfóricos mostra-nos que os diésteres glicero-difosfóricos apresentam uma percentagem de fósforo superior à dos outros ésteres. Sendo, como já dissemos, os glicerofosfatos indicados em todos os casos que há necessidade de aumentarmos a taxa de fósforo orgânico, facilmente se infere o porquê da nossa defesa pela amostra V.

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CONCLUSÕES

1) Desejamos ressaltar os estudos de Marfori, Maestro e Cartaldi a respeito da não absorção do ácido glicerofosfórico sintético, quando introduzido por via parenteral.
2) O processo volumétrico de Astruc associado aos ensaios qualitativos permite-nos exercer um contrôle satisfatório do glicerofosfato de cálcio oficinal.

3) Entre os ensaios qualitativos não podemos deixar os limites de alcalinidade e de acidez, assim como a pesquisa do ácido cítrico.

4) O paradigma que estabelecemos para contrôle do glicerofosfato de cálcio nos pode orientar com mais precisão, se o produto satisfizer ou não as exigências requeridas.

5) As provas, que apresentamos contra as chamadas soluções de glicerofosfato de cálcio a 50%, são suficientes para que as autoridades competentes providenciem a respeito.

6) Precisamos alargar em nosso código farmacêutico o limite de solubilidade do glicerofosfato de cálcio oficinal.

7) Os farmacologistas devem provar experimentalmente, se os diésteres-glicero-difosfóricos são ou não absorvidos pelo organismo.

8) Caso fique provado que o suco pancreático não consegue cindir êsses diésteres, temos necessidade de estabelecer um limite dos mesmos em o glicerofosfato de cálcio oficinal.

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SYRUP OF GLYCEROPHOSPHATE COMPOUND
Method of Analysis

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Composition

F.U. sodium glycerophosphate	6 g
Commercial magnesium glycerophosphate	2 g
F.U. Calcium glycerophosphate	2 g
Commercial ferrous glycerophosphate	1 g
Detannified liquid extract of kola nut	5 g
Sugar	110 g
water	q.b.a. 200 g
200 g flask	(INAM formula no. 241)

Characteristics

Limpid syrup, yellowish in color. Thickness, 1.28-1.32.

Identification

a) 2 ml of syrup are evaporated and calcined. The residue imparts a yellow color and a brick red color to the flame.

b) 10 ml of water are added to 2 ml syrup. 1 ml of acetic acid at 96% and 5 ml of ammonium oxalate solution at 4%; a white precipitate is obtained.

c) 10 ml of water are added to 2 ml syrup. 2 ml of ammonia at 32% and 5 ml of bibasic sodium phosphate solution at 10%; a white precipitate is obtained.

d) 5 ml of water are added to 2 ml syrup. 5 ml of hydrochloric acid at 8%. 2 ml chlorhydrate hydroxylamine solution at 5 %, 5 ml ammonium acetate solution at 30% and 2 ml α,α-dipyridil at 5% in ethyl alcohol 95%; a red color is obtained.

e) To the residue obtained in a) there are added 5 ml of water and 2 ml nitric acid at 65% and this is filtered. 5 ml of ammonium molibdate solution at 5% are added to the filtrate and are heated; a yellow precipitate is obtained.

f) 10 ml sulfuric acid at 20% are added to 50 ml of syrup and this is extracted with 20 ml ethyl ether. The ether solution is eliminated, the rest is alkalized with ammonia at 10% and ex-

tracted with 3 portions of 20 ml each of chloroform. The chloroform solutions are combined, they are anhydrated in sodium sulfate anhydride and the solvent is evaporated at b.m. 1 ml hydrochloric acid at 35% is added to the residue. Also added are 0.1 g potassium chlorate, and the sum is evaporated dry at b.m. When the residue obtained was exposed to ammonia vapors, it assumed a purplish-red color.

Quantitative determinations

a) Calcium glycerophosphate

To about 15 g of syrup, weighed exactly, are added 50 ml water, 2 ml acetic acid at 96% and this is heated to boiling; 20 ml of ammonium oxalate solution at 4% are added, left at b.m. for one hour and then filtered. The precipitate is washed several times with hot water, it is transferred entirely to a beaker with 50 ml water, acidified with 20% sulfuric acid and titrated at a temperature of 70° with a solution of 0.1 N of potassium permanganate. At the end of the titration the filtrate is immersed for 30 seconds and the titration is continued.

$$\text{g of } CaC_3H_5(OH)_2PO_4 \cdot 2H_2O \text{ in 200 g of syrup} = \frac{n \cdot 0.0123}{p} \cdot 200$$

where:

n = ml of $KMnO_4$ 0.1 N used in the titration
p = grams of syrup used
0.0123 = grams of $CaC_3H_5(OH)_2PO_4 \cdot 2H_2O$ equivalent to 1 ml $KMnO_4$ 0.1 N

b) Ferrous glycerophosphate

About 10 g of syrup, weighed exactly, are diluted with water at 250 ml. To 10 ml in a marked flask of 100 ml capacity are added 5 ml hydrochloric acid at 8%, 10 ml of hydroxylamine chlorhydrate at 5%; after 5 minutes 10 ml of ammonium acetate solution at 30%; after 5 minutes 4 ml of α,α-dipyridil solution at 0.5% in 95% ethyl alcohol, and this is diluted in volume with water. After one hour a reading is taken of 1 cm with a colorimeter or spectrophotometer with a wave length of 522 mμ in contrast to a white test.

$$\text{g of } Fe_2|C_3H_5(OH)_2PO_4|_3 \text{ in 200 g syrup} = \frac{n \cdot 0.005567 \cdot 25}{p} \cdot 200$$

where:

n = mg of Fe deducted from the sample curve
p = grams of syrup used
0.005567 = grams of $Fe_2|C_3H_5(OH)_2PO_4|_3$ equivalent to 1 mg Fe.

(The ferrous glycerophosphate contains from 73 to 83 percent anhydridated product).

c) Magnesium glycerophosphate

About 10 g of syrup, weighed exactly, are diluted with 250 ml water. To 50 ml in a separating column are added 5 ml hydrochloric acid at 8%, 10 ml of potassium sulfocyanate solution at 5%, and this is extracted with three portions of 20 ml each of a mixture of equal parts of iso-amyl alcohol and ethyl ether. The ether-alcohol solution is washed with two portions of 10 ml each of water. The washing water is added to the acqueous solution, this is washed with 10 ml of a mixture of iso-amyl alcohol and ethyl ether and heated at b.m. until the possibly present solvent disappears. There are added 20 ml of a buffer solution obtained by mixing five parts ammonia N with one part of a solution N of ammonium chloride, and this is titrated with a 0.01 M solution of sodium editacetate (EDTA).

$$\text{g of } MgC_3H_5(OH)_2PO_4 \cdot 2H_2O \text{ in 200 g of syrup} = \left[n \cdot \frac{0.002304 \cdot 5}{p} \cdot \left[200 \right] - 0.9365 \cdot A \right.$$

where:

n = ml of EDTA 0.01 M used in the titration

p = grams of syrup used

A = grams of $CaC_3H_5(OH)_2PO_4 \cdot 2H_2O$ determined in 200 g syrup according to a).

0.9365 = grams of $MgC_3H_5(OH)_2PO_4 \cdot 2H_2O$ equivalent to 1 g $CaC_3H_5(OH)_2PO_4 \cdot 2H_2O$

0.002304 = grams of $MgC_3H_5(OH)_2PO_4 \cdot 2H_2O$ equivalent to 1 ml EDTA 0.01 M.

d) Total glycerophosphates

To about 1 g syrup, weighed exactly in a porcelain crock, are added 1 g magensium oxide and this is calcined. After cooling, there are added 5 ml water, 5 ml perhhloric acid at 70%, this is evaporated at b.m. and heated on a direct flame until the white fumes disappear. This is then cooled, the residue is diluted with 6 ml hydrochloric acid at 35%; this is transferred to a beaker using 10 ml water and 15 of hydrochloric acid at 35%. There are added 30 ml of sodium molibdate solution at 15% and this is heated to boiling. 80 ml of quinoline solution at 2% in 2.5% hydrochloric acid are added, boiling is protracted for a few minutes, the solution is filtered and the precipitate washed with 50 ml N hydrochloric acid and with water until there is a neutral reaction. The precipitate is transferred to a beaker with the help of a 0.5 N solution of sodium hydroxide and titrated with hydrochloric acid 0.5 N (indicator, 0.15 ml of timol blue solution at 0.04% in ethyl alcohol at 95% and 0.05 ml of phenolphthalein solution at 1% in 90% alcohol).

When a yellow color is obtained, the filtrate is immersed and titration is continued.

g of total glycerophosphates in 200 g of syrup =

$$\frac{(m \cdot n) \cdot 0.0005961 \cdot 8.835}{p} \cdot 200$$

where:

m = 50 ml of 0.5 N solution of sodium hydroxide
n = ml of hydrochloric acid 0.5 N used in the titration
p = grams of syrup used
0.0005961 = grams of P equivalent to 1 ml 0.5 N solution of sodium hydroxide
8.835 = grams of total glycerophosphates equal to 1 g of P.

e) Caffein (from the liquid extract of detannified cola nut)

About 20 g of syrup, weighed exactly, are transferred into a separator, 25 ml sulfuric acid at 20% are added, and this is extracted with two successive portions of 20 ml each of ethyl ether. The ethereal liquids are washed with 10 ml water; the acqueous solutions are combined and alkalized with 32% ammonia and extracted with six portions of 200 ml each of chloroform.

The chloroform solutions are washed with 20 ml N sulfuric acid, anhydrated in sodium sulfate anhydride, transferred into a marked flask of 200 ml capacity and diluted with chloroform. 10 ml are withdrawn, diluted with 50 ml chlororform and a reading is taken of ultra-violet absorption on 1 cm at a wave length of 276 mμ and 310 mμ.

$$\text{mg of } C_8H_{10}O_2N_4 \cdot H_2O \text{ in 200 g syrup} = \frac{(E_1 \cdot E_2) \cdot 10}{0.480 \cdot p} \cdot 200$$

where:
E_1 = extinction read at 276 mμ.
E_2 = extinction read at 310 mμ.
p = grams of syrup used
0.480 = $E^{1\%}_{1cm}$ (specific extinction of the solution of 1 mg caffein in 100 ml chloroform in 1 cm).
(the liquid kola nut extract contains 1 percent caffein).

f) Sugar (saccharose)

About 10 g of syrup, weighed exactly, are diluted with 100 ml water. A polarimetric reading with light in sodium at 20° then is taken.

$$\text{g of saccharose in 200 g of syrup} = \frac{\alpha \cdot 100}{l \cdot 66^\circ} \cdot 20$$

where:

α = angle of rotation obtained.
l = length of the polarimetric tube expressed in dm.
66° = specific rotatory power.

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NOTE PRATICHE

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SCIROPPO DI GLICEROFOSFATI COMPOSTO F. G.
Metodo d'analisi

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Composizione

Sodio glicerofosfato F.U.	6 g
Magnesio glicerofosfato officinale	2 g
Calcio glicerofosfato F.U.	2 g
Ferro glicerofosfato officinale	1 g
Estratto fluido noce cola detannizzato	5 g
Zucchero	110 g
Acqua	q.b.a. 200 g

Flacone da 200 g — *Formula INAM n. 2410*

Caratteri

Sciroppo limpido di colore giallognolo. Densità 1.28-1.32.

Identificazione

a) 2 ml di sciroppo si evaporano e si calcinano. Il residuo impartisce alla fiamma colorazione gialla e colorazione rosso mattone.

b) A 2 ml di sciroppo si aggiungono 10 ml di acqua, 1 ml di acido acetico al 96 per cento e 5 ml di soluzione di ammonio ossalato al 4 per cento: si ottiene precipitato bianco.

c) A 2 ml di sciroppo si aggiungono 10 ml di acqua, 2 ml di ammoniaca al 32 per cento e 5 ml di soluzione di sodio fosfato bibasico al 10 per cento: si ottiene precipitato bianco.

d) A 2 ml di sciroppo si aggiungono 5 ml di acqua, 5 ml di acido cloridrico all'8 per cento, 2 ml di soluzione di idrossilamina cloridrato al 5 per cento, 5 ml di soluzione di ammonio acetato al 30 per cento e 2 ml di soluzione di *α, α*-dipiridile al 5 per cento in alcole etilico 95°: si ottiene colorazione rossa.

e) Al residuo ottenuto secondo *a*) si aggiungono 5 ml di acqua e 2 ml di acido nitrico al 65 per cento e si filtra. Al filtrato si aggiungono 5 ml di soluzione di ammonio molibdato al 5 per cento e si riscalda: si ottiene precipitato giallo.

f) A 50 ml di sciroppo si aggiungono 10 ml di acido solforico al 20 per cento e si estrae con 20 ml di etere etilico. Si elimina la soluzione eterea, si alcalinizza con ammoniaca al 10 per cento e si estrae con 3 porzioni da 20 ml ciascuna di cloroformio. Si riuniscono le soluzioni cloroformiche, si anidrificano su sodio solfato anidro e si evapora il solvente a b. m.. Al residuo si aggiungono 1 ml di acido cloridrico al 35 per cento, 0.1 g di potassio clorato e si evapora

a secchezza a b. m.. Il residuo ottenuto esposto a vapori ammoniacali, assume colorazione rosso porpora.

Determinazioni quantitative

a) Calcio glicerofosfato

A 15 g circa di sciroppo, esattamente pesati, si aggiungono 50 ml di acqua, 2 ml di acido acetico al 96 per cento e si riscalda all'ebollizione; si aggiungono 20 ml di soluzione di ammonio ossalato al 1 per cento, si lascia a b. m. per un'ora e si filtra. Si lava il precipitato più volte con acqua calda, si trasferisce quantitativamente in bicchiere con 50 ml di acqua, si acidifica con acido solforico al 20 per cento e si titola alla temperatura di 70° con soluzione 0,1 N di potassio permanganato. Al termine della titolazione si immerge il filtro per 30 secondi e si continua la titolazione.

$$g \text{ di } CaC_3H_5(OH)_2PO_4.2H_2O \text{ in } 200 \text{ g di sciroppo} = \frac{n \cdot 0{,}0123}{p} \cdot 200$$

dove:

n = ml di $KMnO_4$ 0,1 N impiegati nella titolazione.

p = grammi di sciroppo usati.

0,0123 = grammi di $CaC_3H_5(OH)_2PO_4 . 2 H_2O$ equivalenti a 1 ml di $KMnO_4$ 0,1 N.

b) Ferro glicerofosfato

10 g circa di sciroppo, esattamente pesati, si diluiscono con acqua a 250 ml. A 10 ml, in matraccio tarato da 100 ml, si aggiungono 5 ml di acido cloridrico all'8 per cento, 10 ml di soluzione di idrossilamina cloridrato al 5 per cento; dopo 5 minuti 10 ml di soluzione di ammonio acetato al 30 per cento; dopo 5 minuti 1 ml di soluzione di α, α-dipiridile allo 0,5 per cento in alcole etilico a 95° e si diluisce a volume con acqua. Dopo un'ora si effettua la lettura in celle da 1 cm con colorimetro o spettrofotometro alla lunghezza d'onda di 522 mμ in confronto alla prova in bianco.

$$g \text{ di } Fe_2[C_3H_5(OH)_2PO_4]_3 \text{ in } 200 \text{ g di sciroppo} = \frac{n \cdot 0{,}005567 \cdot 25}{p} \cdot 200$$

dove:

n = mg di Fe dedotti dalla curva campione.

p = grammi di sciroppo usati.

0,005567 = grammi di $Fe_2[C_3H_5(OH)_2PO_4]_3$ equivalenti a 1 mg di Fe.

(Il ferro glicerofosfato officinale contiene dal 73 all'83 per cento di prodotto anidro).

c) Magnesio glicerofosfato

10 g circa di sciroppo, esattamente pesati, si diluiscono con acqua a 250 ml. A 50 ml in palloncino separatore, si aggiungono 5 ml di acido cloridrico all'8 per cento, 10 ml di soluzione di potassio solfocianato al 5 per cento e si estrae con tre porzioni da 20 ml ciascuna di una miscela di volumi eguali di alcole

iso-amilico ed etere etilico. La soluzione eterea-alcoolica si lava con due porzioni da 10 ml ciascuna di acqua. Le acque di lavaggio riunite alla soluzione acquosa si lavano con 10 ml di miscela di alcole iso-amilico ed etere etilico e si riscaldano a b. m. sino a scomparsa dell'eventuale solvente. Si aggiungono 20 ml di soluzione tampone ottenuta miscelando cinque volumi di ammoniaca N con un volume di soluzione N di ammonio cloruro e si titola con soluzione 0.01 M di editacetato sodico (EDTA).

g di $MgC_3H_5(OH)_2PO_4 \cdot 2H_2O$

$$\text{in 200 g di sciroppo} = \left[\frac{n \cdot 0.002304 \cdot 5}{p} \cdot 200 \right] \cdot 0.9365 - A.$$

dove:

n = ml di EDTA 0.01 M impiegati nella titolazione.

p = grammi di sciroppo usati.

A = grammi di $CaC_3H_5(OH)_2PO_4 \cdot 2H_2O$ determinati in 200 g di sciroppo secondo a).

0.9365 = grammi di $MgC_3H_5(OH)_2PO_4 \cdot 2H_2O$ equivalenti a 1 g di $CaC_3H_5(OH)_2PO_4 \cdot 2H_2O$.

0.002304 = grammi di $MgC_3H_5(OH)_2PO_4 \cdot 2H_2O$ equivalenti a 1 ml di EDTA 0.01 M.

d) Glicerofosfati totali

A 1 g circa di sciroppo esattamente pesato in crogiolo di porcellana si aggiunge 1 g di magnesio ossido e si calcina. Dopo raffreddamento si aggiungono 5 ml di acqua, 5 ml di acido perclorico al 70 per cento, si evapora a b. m. e si riscalda a fiamma diretta sino a scomparsa dei fumi bianchi. Si raffredda, si scioglie il residuo con 6 ml di acido cloridrico al 35 per cento; si trasferisce quantitativamente in bicchiere con l'aiuto di 10 ml di acqua e 15 di acido cloridrico al 35 per cento. Si aggiungono 30 ml di soluzione di sodio molibdato al 15 per cento e si riscalda all'ebollizione. Si aggiungono 30 ml di soluzione di chinolina al 2 per cento in acido cloridrico al 2.5 per cento, si protrae l'ebollizione per qualche minuto, si filtra e si lava il precipitato con 50 ml di acido cloridrico N e con acqua sino a reazione neutra. Si trasferisce quantitativamente il precipitato in bicchiere con l'aiuto di 50 ml di soluzione 0.5 N di sodio idrossido e si titola con acido cloridrico 0.5 N (indicatore 0.15 ml di soluzione di blu timolo allo 0.04 per cento in alcol etilico a 95° e 0.05 ml di soluzione di fenolftaleina all'1 per cento in alcole a 90°).

Quando si ottiene colorazione gialla si immerge il filtro e si continua la titolazione.

$$\text{g di Glicerofosfati totali in 200 g di sciroppo} = \frac{(m-n) \cdot 0.0005961 \cdot 8.835}{p} \cdot 200$$

dove:

m = 50 ml di soluzione 0.5 N di sodio idrossido.

n = ml di acido cloridrico 0.5 N impiegati nella titolazione.

p = grammi sciroppo usati.

0,0005961 = grammi di P equivalenti a 1 ml di soluzione 0,5 N di idrossido di sodio.

8,835 = grammi di glicerofosfati totali equivalenti a 1 grammo di P.

e) **Caffeina** (dall'estratto fluido noci di cola detannizzato)

20 g circa di sciroppo, esattamente pesati, si trasferiscono in separatore, si aggiungono 25 ml di acido solforico al 20 per cento e si estrae con due successive porzioni da 20 ml ciascuna di etere etilico. I liquori eterei si lavano con 10 ml di acqua; le soluzioni acquose riunite si alcalinizzano con ammoniaca al 32 per cento e si estraggono con sei porzioni da 20 ml ciascuna di cloroformio.

Le soluzioni cloroformiche si lavano con 20 ml di acido solforico N, si anidrificano su sodio solfato anidro, si trasferiscono in matraccio tarato da 200 ml e si diluiscono a volume con cloroformio. Si prelevano 10 ml, si diluiscono a 50 ml con cloroformio e si esegue la lettura dell'assorbimento nell'u. v. in celle da 1 cm alla lunghezza d'onda di 276 mμ e 310 mμ.

$$\text{mg di } C_8H_{10}O_2N_4 \cdot H_2O \text{ in 200 g di sciroppo} = \frac{(E_1 - E_2) \cdot 10}{0{,}480 \cdot p} \cdot 200$$

dove:

E_1 = estinzione letta a 276 mμ.

E_2 = estinzione letta a 310 mμ.

p = grammi di sciroppo usati.

0,480 = $E^{1\%}_{1\,cm}$ (estinzione specifica della soluzione di 1 mg di caffeina in 100 ml di cloroformio in celle da 1 cm.

(l'estratto fluido noce di cola contiene 1 per cento di caffeina).

f) **Zucchero** (saccarosio)

10 g circa di sciroppo, esattamente pesati si diluiscono a 100 ml con acqua. Si esegue la lettura polarimetrica con luce al sodio a 20°.

$$\text{g di saccarosio in 200 g di sciroppo} = \frac{\alpha \cdot 100}{l \cdot 66^\circ} \cdot 20$$

dove:

α = angolo di rotazione ottenuto.

l = lunghezza del tubo polarimetrico espressa in dm.

66° = potere rotatorio specifico.

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STUDY OF LOCAL EFFECTS OF CALCIUM GLYCEROPHOSPHATE AND SODIUM FLUORIDE ON EXPERIMENTAL DENTAL CARIES IN WHITE RATS

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The anti-caries effect of phosphates introduced into an organism with food has been conclusively pointed out in studies by both Russian and foreign authors (4b,c; 7, 12, 13). Our clinical observations confirmed the beneficial effect accessible by means of endogenic and associated (endogenic and local) application of organic preparations of phosphorus (calcium glycerophosphate, phosphren, and phytin) during the treatment of systemic hyperaesthesia of dentin (4d, 5).

Data are found in the literature on the local effect of phosphates on softened dentin and the frequency of experimental dental caries (2, 10, 11). Several investigators note an increase in the anti-caries activity of phosphates during their simultaneous local effect on hard tooth tissue with sodium fluoride (16, 18). However some of these studies were conducted in vitro and the data obtained by the authors are inconclusive.

The present work was undertaken with the aim of studying the possibility for the local effect of phosphorus and fluorine preparations, introduced in toothpaste, on experimental dental caries in rats. The conflicting data on the local effect of fluorine (1,3,7,14,15) awakened us to the comparison of the anti-caries effect of phosphates and fluorine during their local application.

The study was conducted on white rats of a single litter of the Vistar strain. Four groups of rats were under observation (58 animals) which were kept on a carbohydrate caries-forming diet (4b) for 30 days. Animal groups I, II, and III underwent, from the instant of their transfer to the caries-forming diet, a single daily hygienic cleaning of their teeth with different toothpastes - phosphate, sodium fluoride, and an ordinary one, using a specially prepared toothbrush, followed by a careful washing out of the mouth cavity (in order to keep toothpaste from falling into the gastro-intestinal tract). The tooth cleaning with paste was done at the end of the day, after which no food was given.

The control animals (group IV) received only a caries-forming ration and did not undergo cleaning. None of the toothpastes contained antiseptic or aromatic substances. Into the makeup of the toothpastes went Na carboxyl-methyl-cellulose, which possesses good cleaning properties and which increases ion exchange processes.

Thus, the animals were grouped as follows:

I: Rats' teeth cleaned with a paste consisting of 2.5 % calcium glycerphosphate, 2 % carboxyl-methyl-cellulose, 29.6 % chalk, 43 % water, 20 % glycerine, and 2.9 % table salt.

II: Rats' teeth cleaned with a paste composed of 0.1 % sodium fluoride (in accordance with recommendations in the literature, such a concentration is completely reliable and does not cause an obvious intoxication in the organism), 2 % carboxyl-methyl-cellulose, 44.2 % chalk, 26.9 % glycerine, and 26.9 % water.

III: Rats' teeth cleaned with an ordinary toothpaste composed of 1.2 % carboxyl-methyl-cellulose, 43.4 % chalk, 27.7 % water, and 27.7 % glycerine.

IV: Rats whose teeth were not cleaned.

For a time 93-95 days from birth and 60 days on a caries-forming ration, the animals were decapitated and a blood sample was taken to study inorganic phosphorus (using Briggs' method) and calcium (using De Waard's method) in the blood serum. Then the jaws of the animals were cleaned of

soft tissue and were studied under a binocular microscope to determine the number and nature of caries disease occurrences.

TABLE 1. VULNERABILITY TO CARIES IN ANIMALS FROM THE EXPERIMENTAL GROUPS

Group	Mean wt of rats, g (M ± m)	Anti-caries agent in pastes	Total # animals	# animals free of caries	Aver. # caries occurr. in one rat
I	112.0 + or - 7.4	2.5 % calcium glycerophosphate	11	3	3.0 + or - 0.68
II	103.6 + or - 7.8	0.1 % sodium fluoride	11	-	5.3 + or - 0.81
III	109.8 + or -12.6	-	10	-	5.9 + or - 0.76
IV	139.4 + or - 5.0	Control	26	-	6.3 + or - 0.56

The results of the experiments conducted showed that even a single daily hygienic care of the teeth with any of the pastes named deceased somewhat the frequency of caries disease and altered its character. However a minimum number of caries occurrences were present in animals treated with toothpaste containing calcium glycerophosphate (Table 1).

TABLE 2. PROBABILITY OF VARIANCE BETWEEN FREQUENCY OF DENTAL CARIES DISEASE IN ANIMALS OF THE DIFFERENT GROUPS

Group	Group I	Group II	Group III
IV	$P < 0.001$	$P > 0.2$	$P > 0.5$
III	$P < 0.02$	$P > 0.5$	
II	$P < 0.05$		

As is evident from the table, the maximum number of caries occurrences was in the control group (6.3 + or - 0.56).

The mean number of caries occurrences in one animal in the groups of rats using toothpaste containing sodium fluoride (II) and not including an anti-caries agent (III), was somewhat less than in the control group. However, statistically reliable differences could not be found with the comparison of the results obtained (Table 2).

It is characteristic that only group I (animals which used toothpaste with calcium glycerophosphate) showed a high reliability of variance in the frequency of dental caries disease in comparing these data with the results obtained for rats in groups II, III, and IV. The fact must also be noted that caries are absent in three animals from group I, which is an extremely reliable indicator of the prophylactic effect of phosphates.

In spite of the absence of reliable differences in frequency of dental caries disease in groups II, III, and IV, its severity in the separate experimental groups was different (Table 3).

Thus, in group II, 44.8 % of the animals had caries disease of the enamel and dentin, 59.3 % in group III, and in the control group 59.5 % had moderate and deep caries disease. In group I, 39.3 % of the animals had such caries occurrences. Comparing these results with those obtained by us previously in a study of the endogenic effect of phosphates and sodium

fluoride (4b), it must be noted that the local anti-caries effect of the preparations indicated is significantly less, since the number of deep and moderate caries occurrences remains high in the given case.

TABLE 3. CHARACTERISTICS OF CARIES DISEASE IN ANIMALS OF DIFFERENT EXPERIMETAL GROUPS

Group	Anti-caries agent in pastes	Total caries occurr.	Degree of caries disease		
			Incip. caries	Caries of enamel	Caries of dentin
I	2.5 % calcium glycerophosphate	33	20	5	8
II	0.1 % sodium fluoride	58	32	18	8
III	-	59	24	20	15
IV	Control	163	66	55	42

A significant difference in dental disease was not noted in males and females. A relatively high frequency of disease of the lower teeth (3-4 times, on the average) not only confirms the data from the literature on this question, but indicates a definite characteristic in the development of caries during the action of local anti-caries agents. In these situations the upper teeth were diseased still less often than during the administration of phosphorus and fluoride preparations with feed.

TABLE 4. CONTENT OF INORGANIC PHOSPHORUS AND CALCIUM IN BLOOD SERUM IN EXPERIMENTAL ANIMALS (M + m)

Group	Anticaries agent in toothpaste	Phosphorus	P	Calcium	P
I	2.5 % calcium glycerophosphate	9.20 + or - 0.52	$P_{I-II} > 0.5$	9.53 + or - 0.68	$P_{I-II} > 0.1$
II	0.1 % sodium fluoride	9.36 + or - 0.32	$P_{I-III} > 0.2$	10.86 + or - 0.74	$P_{I-III} > 0.05$
III	-	9.72 + or - 0.30	$P_{II-III} > 0.5$	10.88 + or - 0.41	$P_{II-III} > 0.5$

The study of the amount of inorganic phosphorus and calcium in blood serum of rats in the three experimental groups showed that, in the animals studied, there is no significant difference in the amounts of these elements (Table 4).

It is thoroughly understood that the absence of a significant difference in frequency of dental caries disease and especially local application of anti-caries agents may not be reflected in the phosphorus and calcium content in blood serum, as the data presented in Table 4 confirm.

Thus, the studies conducted permit us to establish that the most active anti-caries component in toothpaste (of the three types studied) is calcium glycerophosphate, which evidently proves to have a topical effect due to an increase in the mineral lattices of the enamel by activating and accelerating ion exchange processes taking place in the mouth cavity (4a, 8, 17). Introduction into the toothpaste receptor of Na carboxyl-methyl-cellulose, an active ion exchange medium, must have been aided by these processes.

Sdoium fluoride, which manifests a definite anti-caries effect during internal administration in toothpaste, was not very effective, as the

experimental data indicated. This is in contradiction to a number of other studies (6,7), whose authors recommend its substitution for other salts: stannous fluoride or calcium fluoride.

The data obtained on the topical anti-caries effect of calcium glycerophosphate permit the suggestion that the combination of general and local effects of phosphorus preparations would yield the possibility of obtaining a still greater reduction in the frequency of caries disease. Further experimental studies in this direction might yield practical recommendations for dental caries prophylactics.

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ЭКСПЕРИМЕНТАЛЬНАЯ МОРФОЛОГИЯ

Ю. А. ФЕДОРОВ

ИЗУЧЕНИЕ МЕСТНОГО ДЕЙСТВИЯ ГЛИЦЕРОФОСФАТА КАЛЬЦИЯ И ФТОРИСТОГО НАТРИЯ НА ЭКСПЕРИМЕНТАЛЬНЫЙ КАРИЕС ЗУБОВ У БЕЛЫХ КРЫС

(Представлено академиком Н. Н. Аничковым 22 VIII 1964)

Противокариозное действие фосфатов, вводимых в организм с пищей, убедительно показано в некоторых исследованиях отечественных и зарубежных авторов ([4,5, 6, 7, 12, 13]). Наши клинические наблюдения подтвердили хороший эффект, достигаемый путем эндогенного и сочетанного (эндогенного и местного) применения органических препаратов фосфора (глицерофосфат кальция, фосфрен, фитин) при лечении системной гиперестезии дентина ([4г, 5]).

В литературе имеются данные и о местном действии фосфатов на размягченный дентин и частоту экспериментального кариеса зубов ([2, 10, 11]). Некоторые исследователи отмечают увеличение противокариозной активности фосфатов при их совместном локальном воздействии на твердые ткани зуба с фтористым натрием ([16, 18]). Однако часть из этих исследований была проведена in vitro, и данные, полученные авторами, неубедительны.

Настоящая работа предпринята с целью изучения возможности местного воздействия препаратами фосфора и фтора, вводимыми в составе зубных паст, на экспериментальный кариес зубов у крыс. Противоречивость данных о местном действии фтора ([1, 3, 7, 14, 15]) побудила нас к сопоставлению противокариозного эффекта фосфатов и фтора при их местном применении.

Исследование проводилось на белых крысах одного помета линии Вистар. Под наблюдением находилось 4 группы белых крыс (58 животных), содержащихся на углеводной кариесогенной диете ([16]) с 30 дня жизни. Животным I, II, III групп с момента перевода их на кариесогенную диету ежедневно производили одноразовую гигиеническую очистку зубов различными пастами — с фосфатом, фтористым натром и обычной при помощи специально изготовленной зубной щетки, с последующим тщательным промыванием полости рта (чтобы исключить попадание зубной пасты в желудочно-кишечный тракт). Обработку зубов пастой проводили обычно в конце дня, и корм после этого не давали.

Контрольные животные (IV группа) получали только кариесогенный рацион, и очистку у них не производили. Все зубные пасты не содержали антисептика и ароматических веществ. В рецептуры зубных паст входила Na-карбоксиметилцеллюлоза, обладающая хорошей очистительной способностью и усиливающая ионообменные процессы.

Таким образом, животные были разбиты на следующие группы:

I. Крысы, у которых очистка зубов производилась пастой, содержащей 2,5% глицерофосфата кальция, 2% карбоксиметилцеллюлозы, 29,6% мела, 43% воды, 20% глицерина и 2,9% поваренной соли.

II. Крысы, у которых очистка зубов производилась пастой, содержащей 0,1% фтористого натрия (согласно рекомендаций литературы, та-

кая концентрация вполне достаточна и не вызывает явной интоксикации организма), 2% карбоксиметилцеллюлозы, 44,2% мела, 26,9% глицерина и 26,9% воды.

III. Крысы, у которых очистка зубов производилась обычной зубной пастой, содержащей 1,2% карбоксиметилцеллюлозы, 43,4% мела, 27,7% воды и 27,7% глицерина.

IV. Крысы, у которых очистка зубов не производилась.

По истечении 93 — 95 дней со дня рождения и 60 дней нахождения на кариесогенном рационе животных убивали декапитацией с одновременным

Таблица 1

Поражаемость кариесом животных различных экспериментальных групп ($M \pm m$)

Группа	Средний вес крыс, г	Противокариозные добавки в пастах	Общее число животных	Число животных, свободных от кариеса	Среднее число кариозных поражений на 1 крысу
I	112,0±7,4	2,5% глицерофосфата кальция	11	3	3,0±0,68
II	103,6±7,8	0,1% фтористого натрия	11	—	5,3±0,81
III	109,8±12,6	—	10	—	5,9±0,76
IV	139,4±5,0	Контроль	26	—	6,3±0,56

взятием крови для исследования в сыворотке неорганического фосфора (по методу Бриггса) и кальция (по методу Де Ваарда). Затем челюсти животных очищали от мягких тканей и исследовали под бинокулярной лупой: отмечали число и характер кариозных поражений.

Результаты проведенных исследований показали, что даже однократная ежедневная гигиеническая обработка зубов любой из названных паст несколько снижает частоту кариозных поражений и изменяет характер последних. Однако минимальное число кариозных поражений было у животных, подвергавшихся воздействию зубной пасты с глицерофосфатом кальция (табл. 1).

Как видно из таблицы, максимальное число кариозных поражений было у контрольной группы животных (6,3 ± 0,56).

Среднее число кариозных поражений на одно животное в группах крыс, подвергавшихся воздействию зубных паст, содержащих фтористый натрий (II) и не включающих противокариозных добавок (III), несколько меньше, чем в контрольной группе, однако статистически достоверного различия при сопоставлении полученных результатов обнаружить не удается (табл. 2).

Таблица 2

Вероятность различия при сопоставлении частоты поражения зубов кариесом у животных различных групп

Группа	Группы		
	I	II	III
IV	$P < 0{,}001$	$P > 0{,}2$	$P > 0{,}5$
III	$P < 0{,}02$	$P > 0{,}5$	
II	$P < 0{,}05$		

Характерно, что только I группа животных (подвергавшихся воздействию пасты с глицерофосфатом кальция) показала высокую достоверность различия в частоте поражения зубов кариесом при сопоставлении этих данных с результатами, полученными у крыс II, III, IV групп. Следует также отметить факт отсутствия кариеса у 3 животных I группы, что является весьма существенным показателем профилактического действия фосфатов.

Несмотря на отсутствие достоверного различия в частоте поражения зубов кариесом у животных групп II, III и IV, тяжесть его течения в отдельных экспериментальных группах была различна (табл. 3).

Так, у животных II группы было 44,8% кариозных поражений эмали и дентина, у животных III группы 59,3% кариозных поражений эмали и дентина, а у животных контрольной (IV) группы 59,5% средних и глубоких кариозных поражений. В I группе животных таких кариозных поражений было всего 39,3%. Сопоставляя эти результаты с полученными нами ранее при изучении эндогенного воздействия фосфатов и фтористо-

Таблица 3

Характеристика кариозных поражений у животных различных экспериментальных групп

Группа	Противокариозные добавки в пасты	Всего кариозных поражений	Степень поражения кариесом		
			начальный кариес	кариес эмали	кариес дентина
I	2,5% глицерофосфата кальция	33	20	5	8
II	0,1% фтористого натрия	58	32	18	8
III	—	59	24	20	15
VI	Контроль	163	66	55	42

го натрия ([46]), следует отметить, что местное противокариозное действие указанных препаратов значительно слабее, так как число глубоких и средних кариозных поражений остается в данном случае высоким.

Но отмечается достоверного различия в поражении зубов у самцов и самок. Относительно большая частота поражения нижних зубов (в среднем в 3—4 раза) не только подтверждает имеющиеся по этому вопросу литературные данные, но и указывает на определенные особенности в развитии кариеса при местном воздействии противокариозными агентами. В этих условиях верхние зубы поражаются еще реже, чем при введении препаратов фосфора и фтора с пищей.

Таблица 4

Содержание неорганического фосфора и кальция в сыворотке крови экспериментальных животных ($M \pm m$)

Группа	Противокариозная добавка к пасте	Фосфор	P	Кальций	P
I	2,5% глицерофосфата кальция	9,20±0,52	$P_{I-II} > 0,5$	9,53±0,68	$P_{I-II} > 0,1$
II	0,1% фтористого натрия	9,36±0,32	$P_{I-III} > 0,2$	10,86±0,74	$P_{I-III} > 0,05$
III	—	9,72±0,30	$P_{II-III} > 0,5$	10,88±0,41	$P_{II-III} > 0,5$

Изучение содержания неорганического фосфора и кальция в сыворотке крови крыс трех экспериментальных групп показало, что у исследуемых животных не имеется достоверного отличия в содержании этих элементов (табл. 4).

Вполне понятно, что отсутствие значительного различия в частоте поражения зубов кариесом и сугубо местное применение антикариозных агентов не могли отразиться на содержании фосфора и кальция в сыворотке крови, о чем и свидетельствуют представленные в табл. 4 данные.

Таким образом, проведенные исследования позволили установить, что наиболее активным противокариозным компонентом зубной пасты (из изученных трех образцов) является глицерофосфат кальция, оказывающий свое топическое действие, по-видимому, за счет укрепления минерально-

решетки эмали путем активации и ускорения ионообменных процессов, протекающих в полости рта ([4а, 8, 17]). Введение в рецептуру зубных паст Na-карбоксиметилцеллюлозы — активного ионообменного средства — должно было способствовать этим процессам.

Фтористый натрий, проявляющий определенное противокариозное действие при введении внутрь, в составе зубной пасты, как показали данные эксперимента, малоэффективен. Это находит подтверждение и в ряде других исследований ([6, 9]), авторы которых рекомендуют заменять его другими солями: фтористым оловом или фтористым кальцием.

Полученные данные о выраженном топическом противокариозном эффекте глицерофосфата кальция позволяют предполагать, что сочетание общего и местного действия препаратов фосфора даст возможность добиться еще большего снижения частоты кариозных поражений. Дальнейшие экспериментальные исследования, проведенные в этом направлении, предоставят возможность дать практические рекомендации по профилактике кариеса зубов.

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INFLUENCE OF SUBSTRATE IN VIVO ON BLOOD GLYCEROPHOSPHATASE

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In preceding studies, we have studied several phenomena which might intervene in the enzyme production of animal organs, by determining an alteration of the activity of certain enzymes in the blood; in a general way, we undertook this very complex problem using a technique of altered systems, either by inducing the formation of defensive enzymes by introducing foreign substances into the organism, or by studying the influence of pathological, artificial or clinical conditions on the enzyme activity of the organs and of the blood. However, recently, we have preferred to observe the effect of the injection of physiological substrates on the effectiveness of their enzymes, existing in the blood under normal conditions; that is to say, we have verified whether the introduction of these substances produces an alteration in the ferment level. In the case of the blood cholinesterase of the guinea pig, Jacobsohn and Mirabeau (1) have confirmed an increase in its activity following the injection of acetylcholine, while they have determined the opposite effect (2) as concerns the lipolytic power of the blood after the injection of tributyrine. The injection of fumarate, according to our former observations (3), alco produces a reduction in the activity of fumarico-hydratase in the guinea pig, while the fermentary level of the blood of a dog remains the same.

In this work, we performed subcutaneous injections of 0.1 to 0.5 g potassium glycerophosphate on guinea pigs, and we determined the activity of the blood in phospnatase (pH = 6.9) before the injection and ½ hour after the administration of the substrate; toward this end, we checked, in vitro, the speed of hydrolysis of the same substrate under the effect of blood (hemolyzed) obtained by pricking the heart. After 27 hours, we dosed the mineralized phosphate, at 37°, in the test liquids whose composition is described in the following table, which also indicates the results obtained. Dosage of the mineral phosphate was done with the colorimetric method of W.I. Holman (4), based on that of Fiske and Subbarow, after deproteinization of an aliquote portion of the test liquid with 5% trichloracetic acid. The mineral phosphate content of the blood and that (very small) of the substrate solution were determined in control tests, and are already considered in the values

indicated in the table:

Test Liquid

5 cm^3 potassium glycerophosphate at 4%
5 cm^3 veronal buffer (pH: 7.0)
10 cm^3 hemolyzed guinea pig blood (1:3)

} pH = 6.9, 37°C

(1 cm^3 = 1.3 mg organic P)

mg mineralized P after 24 hours of hydrolysis	Test I	II
before the injection of glycerophosphate:	0.052	0.085
¾ hours after injection of 0.1 g glycerophosphate:	0.062	0.091
" " " " " 0.5 g " " " :	—	0.094

From the above table, we conclude that the subcutaneous administration of glycerophosphate produces an increase in enzyme activity, which rises up to 15% approximately. Successive injections of the same dose prolonged over several days (0.1 g per day) led to the same result.

We will wait to proceed to the discussion of these results and those of earlier works in a more detailed article, in which we will have above all to consider the literature relative to our subject and, in particular, that concerning the pathology of blood phosphatases.

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ARCHIVES PORTUGAISES DES SCIENCES BIOLOGIQUES — TOME VII — SUPPLEMENT II

COMPTES-RENDUS DES SÉANCES DE LA

SOCIÉTÉ PORTUGAISE DE BIOLOGIE

Séance du 20 avril 1944. Presidence de M. FERREIRA DE MIRA

L'INFLUENCE DU SUBSTRATUM *IN VIVO* SUR LA GLYCÉROPHOSPHATASE SANGUINE

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Dans des travaux précédents, nous avons étudié plusieurs phénomènes qui pourraient intervenir dans la production fermentaire des organes animaux, en déterminant dans le sang l'altération de l'activité de certains enzymes: d'une façon générale, on a abordé ce problème très complexe par la technique des systèmes altérés, soit en provoquant la formation de ferments de défense par l'administration de substances étrangères à l'organisme, soit en étudiant l'influence de conditions pathologiques, artificielles ou cliniques sur l'activité enzymatique des organes et du sang; mais récemment nous avons préféré l'observation de l'effet de l'injection de substrates physiologiques sur l'efficacité de leurs enzymes, existant dans le sang dans des conditions normales; c'est-à-dire nous avons vérifié si l'administration de ces substances produit une altération du taux fermentaire. Dans le cas de la cholinestérase sanguine du Cobaye, JACOBSOHN et MIRABEAU (1) ont constaté une augmentation de son activité à la suite de l'injection de l'acétylcholine, tandis qu'ils ont vérifié (2) l'effet contraire quant au pouvoir lipolytique du sang après l'injection de la tributyrine. Celle du fumarate, de son

côté, d'après nos observations antérieurs (3), produit elle aussi chez le Cobaye, la diminution de l'activité de la fumaricohydratase, tandis que le taux fermentaire du sang du Chien se maintient.

Dans ce travail, nous avons pratiqué l'injection sousculanée de 0,1 à 0,5 g de glycérophosphate de potassium chez le Cobaye et nous avons déterminé l'activité du sang en phosphatase (pH = 6,9) avant l'injection et 1/2 heure après l'administration du substratum; dans ce but nous avons contrôlé, *in vitro*, la vitesse de l'hydrolyse du même substratum sous l'action du sang (hémolysé) obtenu par piqûre du cœur; après 27 heures nous avons dosé le phosphate minéralisé, à 37°, dans les liqueurs d'essai dont la composition est inscrite dans le tableau suivant, qui contient également les résultats obtenus. Le dosage du phosphate minéral a été fait par la technique colorimétrique de W. I. M. Holman (4), basée sur celle de Fiske et Subbarow, après déprotéinisation d'une partie aliquote de la liqueur d'essai avec de l'acide trichloracétique à 5 p. 100. Le contenu du sang en phosphate minéral et celui (très petit) de la solution du substratum ont été déterminés dans des essais de contrôle et sont déjà considérés dans les valeurs inscrits au tableau:

Liqueur d'essai

5 cm³ de glycérophosphate de potassium à 4 p. 100
5 cm³ de tampon véronal (pH : 7,0)
10 cm³ de sang de Cobaye hémolysé (1 : 3)
} $p_H = 6,9$, 37° C

(1 cm³ = 1,3 mg P organique)

mg P minéralisé après 24 heures d'hydrolyse — Essai	I	II
avant l'injection du glycérophosphate:	0,052	0,085
3/4 h. après l'injection de 0,1 g de glycérophosphate:	0,062	0,091
» » » » de 0,5 g » » :	—	0,094

Du tableau ci-dessus on conclut que l'administration souscutanée du glycerophosphate produit une augmentation

de l'activité fermentaire, qui s'élève jusqu'à 15 %, à peu près. Des injections successives de la même dose prolongées pendant plusieurs jours (0.1 g par jour) ont conduit au même résultat.

Nous nous réservons de proceder à la discussion des résultats de cette note et de ceux des notes précédents dans un travail plus détaillé dans lequel il nous faut considérer surtout la litérature relative à notre sujet et, en particulier, celle concernant la pathologie des phosphatases sanguines.

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Succinate, glycerophosphate and ascorbate as sources of cellular energy and as antiteratogens

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SUMMARY

Our experimental findings show that succinate and ascorbate greatly reduce the teratogenic effects of 3-acetylpyridine, 6-aminonicotinamide and sulfanilamide. Glycerophosphate led to similar alleviating results when used in combination with 3-acetylpyridine and 6-aminonicotinamide, but not with sulfanilamide. With certain other teratogens the high-energy intermediates failed to alleviate; in some instances (acetazolamide, insulin) they even led to potentiation of teratogen-induced defects.

The results of our experiments demonstrate clearly that high-energy intermediates, by being fed into the respiratory chain of the mitochondria, can alleviate incidence and degree of expression of malformations produced by specific teratogens. In concert with earlier evidence on the nature of antiteratogenic compounds it can further be concluded that the particular teratogens in question exert their effects by interference with mitochondrial energy production in the tissues for which they have specific affinity.

INTRODUCTION

As a key to teratogenic action a study of antiteratogens is probably at present most likely to yield fruitful results if there is proof or presumptive knowledge that a specific teratogen interferes with the utilization of Krebs cycle intermediates or of steps in hydrogen transfer and phosphorylation and if the corresponding antiteratogens correct this interference. This presumption is derived from a long series of experiments in which we found that the following compounds, when used as supplements, are effective in preventing, wholly or in part, the teratogenic consequences of a variety of substances injected into the yolk sac of developing chicken eggs on the fourth or fifth day of incubation, namely, glucose, pyruvate, tryptophan, α-ketoglutaric acid, 3-hydroxyanthranilic acid, nicotinamide and ADP (Landauer, 1948, 1949, 1953*a*, *b*, 1957; Landauer & Rhodes, 1952; Landauer & Wakasugi, 1967, 1968; Zwilling & DeBell, 1950); and from the virtually certain conclusion that the common denominator, presumably the only common one, in which these compounds share their antiteratogenic activity is related to the synthesis of either nicotinamide adenine dinucleotide (NAD) or adenosine triphosphate (ATP) and the subsequent

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production of energy. The question arose, therefore, if this means that the principal, if not only, role played by antiteratogens is that of providing affected tissues with an extra burst of cellular energy, thereby compensating for damage done by the teratogens. In what follows we shall report on experiments which were designed to test the likelihood of this being true by supplementing teratogen treatment with succinate, glycerophosphate and ascorbate. These compounds were chosen because, as will be discussed further on, they are known to be high-energy sources which can be fed into the respiratory chain of mitochondria and are likely to promote the energy-dependent steps of protein and nucleic acid synthesis.

MATERIALS AND METHODS

The eggs used in our present work came from an interline cross of White Leghorn fowl bred by Sykes International Ltd. in Warminster, Wiltshire. All tests were done after 96 h of incubation. At the end of 18 days of incubation the embryos were removed from the shells and inspected for gross abnormalities. The methods of incubation and the experimental procedures were the same as those recorded earlier (Landauer & Wakasugi, 1967). The number of fertile eggs used, post-operative mortality, number of survivors of the eighteenth day of incubation and incidence of any gross defects found among the survivors are recorded in the tables.

The compounds used in our tests and their sources were the following: 3-acetylpyridine, sulfanilamide and DL-α-glycerophosphate hexahydrate disodium salt, Sigma Chemical Co.; L-ascorbic acid, iso-ascorbic acid sodium salt and propylene glycol, British Drug Houses Ltd.; 6-aminonicotinamide, Mann Research Laboratories; insulin, Burroughs Wellcome and Co.; sodium acetazolamide (Diamox), Lederle Laboratories; sodium succinate hexahydrate, Hopkin and Williams Ltd.

The compounds to be tested were dissolved in demineralized water with 0·3% phenol added, except for sulfanilamide and L-ascorbic acid for which propylene glycol (with added phenol) was used as solvent, and insulin which was used as furnished by Burroughs Wellcome and Co. The aqueous solution of sodium ascorbate was adjusted, with a 10% aqueous solution of L-ascorbic acid, to a pH of about 5. All phenol-containing solutions were left prior to use for 4 days at room temperature. The injection of test compounds was always done into the yolk sac and the volume was generally 0·05 ml, though in some instances 0·1 ml. Substances to be used as supplements were either dissolved together with the teratogen in question or as separate solutions; in the latter instance supplement and test injections were made immediately one after the other, the sequence being indicated in the tables. The dosages of teratogens were arrived at by preliminary toxicity tests; the amounts of supplements to be used were those that could in each instance readily be dissolved in 0·05 or 0·1 ml of solvent. Available evidence (e.g. data of Tables 9 and 10) indicates that it

made no significant difference in our results whether teratogen and supplement were used by separate injections or in the same vehicle. There was no evidence of interaction between those compounds which were dissolved together and, within the dosage ranges of our tests, no changes of solubility.

The developmental defects to which the teratogens used in our tests give rise have been described in the following papers: insulin (Landauer, 1947), sulfanilamide (Ancel, 1945; Zwilling & DeBell, 1950), 3-acetylpyridine and 6-aminonicotinamide (Landauer, 1957), acetazolamide (Landauer & Wakasugi, 1967). Details will not be reviewed in the present report.

RESULTS

Tests with sodium succinate

Table 1 presents the results of a test in which chicken embryos were treated with 3-acetylpyridine alone or in combination with sodium succinate. In our original report on the teratogenic effects of 3-acetylpyridine (Landauer, 1957)

Table 1. *The effect of treatment at 96 h of incubation with 0·6 mg/egg (4·95 μM) 3-acetylpyridine either alone or in combination with 7·5 mg/egg (27·76 μM) sodium succinate.*

(Supplement and teratogen injected together in the same vehicle.)

	3-Acetylpyridine	3-Acetylpyridine supplemented with sodium succinate
Treated ...	196	199
Mortality to 18 days (%)	54·1	24·6
Survivors	90	150
Normal appearance (%)	13·3	44·0
Muscular hypoplasia (%)		
Extreme	57·8	24·7
Slight	12·2	22·0
Suggestive (thin tarsometatarsus)	16·7	9·3
Total	86·7	56·0
Short upper beak (%)	16·7	2·0
Crooked neck (%)	2·2	0·7
Miscellaneous defects (%)	0	0·7

we called attention to the fact that, in addition to the major abnormality of muscular hypoplasia, chiefly of the leg muscles, there is also thinning of the long bones. This latter defect, especially thinning of the tarsometatarsal bone, was in some instances clearly discernible when there was doubt whether the leg musculature was normal or not. Hence the condition of the tarsometatarsus served in a small proportion of cases as the means of diagnosis. The data of

Table 1 indicate a highly significant reduction in toxicity, as expressed by embryo mortality, when 3-acetylpyridine was given in combination with sodium succinate ($P \ll 0{\cdot}0001$). Among survivors of the eighteenth day of incubation succinate supplementation led to a great increase in incidence of normal-

Table 2. *The effect of treatment at 96 h of incubation with 5 γ/egg (0·035 μM) 6-aminonicotinamide either alone or in combination with 7·5 mg/egg (27·76 μM) sodium succinate*

(Supplement and teratogen injected together in the same vehicle.)

	6-Aminonicotinamide	6-Aminonicotinamide supplemented with sodium succinate
Treated ...	150	141
Mortality to 18 days (%)	15·3	11·3
Survivors	127	125
Normal appearance (%)	3·9	31·2
Micromelia (%)	96·0	68·8
Abnormal beak (%)	55·1	32·8
Syndactylism (%)	26·0	12·8

Table 3. *The effect of treatment at 96 h of incubation with 1·3 mg/egg (7·55 μM) sulfanilamide either alone or preceded by injection of 7·5 mg/egg (27·76 μM) sodium succinate*

	Sulfanilamide	Sulfanilamide and sodium succinate
Treated ...	183	117
Mortality to 18 days (%)	21·9	31·6
Survivors	143	80
Normal appearance (%)	32·2	58·8
Micromelia (%)		
Extreme	35·7	22·5
Slight	31·5	18.8
Total	67·1	41·3
Abnormal beak (%)	23·8	13·8
Miscellaneous defects (%)	0	2·1

appearing embryos and a lowering in frequency of over-all muscular hypoplasia, of shortening of the upper beak and of abnormality of the cervical vertebrae. The differences between the two groups are highly significant for muscular hypoplasia and abnormality of the beak ($P \ll 0{\cdot}0001$), and this remained true when, to guard against any possible misclassification, account was taken of only the more extreme expressions of muscular hypoplasia.

Table 2 summarizes our data for 6-aminonicotinamide with or without succinate supplementation. In the presence of succinate the embryo mortality

was slightly, but non-significantly, lowered. The incidence of normal-appearing embryos was greatly increased by succinate supplementation, and the frequency of micromelia, of abnormality of the beak and of syndactylism was much reduced. The difference between the two groups was significant ($P < 0{\cdot}01$) in the incidence of syndactylism; the frequencies of micromelia and beak defect, as well as that of gross normality, differed highly significantly ($P < 0{\cdot}0001$).

The results of a test with sulfanilamide and the effect of succinate on its teratogenicity are given in Table 3. As compared with sulfanilamide alone, embryo mortality was somewhat increased in the presence of sodium succinate, but the difference lacked statistical significance ($P > 0{\cdot}05$). Among survivors of the eighteenth day of incubation highly significant differences occurred between the two groups in 'normality' and in incidence of micromelia ($P < 0{\cdot}001$). Lest it be thought that differential mortality accounted for these results, the significance was also calculated on the assumption that in the group treated with sulfanilamide alone, all dead embryos had been non-micromelic, and that all dead embyros had been micromelic in the succinate-supplemented group. The results still demonstrated beyond doubt that the presence of succinate lowered the incidence of micromelia ($P < 0{\cdot}01$). The percentage reduction in incidence of beak defects after succinate supplementation was even greater than that of micromelia (53·4 versus 38·5 %), but the difference did not reach statistical significance.

In earlier experiments (Landauer & Rhodes, 1952) it had been shown that the combination of insulin and sodium succinate caused a high degree of potentiation of all insulin-induced malformations, and this in spite of the fact that treatment with sodium succinate alone was quite non-teratogenic.

Tests with DL-α-*glycerophosphate hexahydrate disodium salt*

We conducted two tests in which the interaction, if any, of 3-acetylpyridine and glycerophosphate was to be examined. Since the two experiments produced closely similar results we combined the data and these are shown in Table 4. It will be seen that the toxicity of 3-acetylpyridine, as expressed in embryo mortality, was greatly reduced in the presence of glycerophosphate and that the incidence of muscular hypoplasia, abnormality of the upper beak and defective formation of the cervical vertebrae was greatly lowered, with a corresponding increase in the frequency of grossly normal embryos. All these differences in toxicity and teratogenicity are statistically highly significant ($P \ll 0{\cdot}0001$).

The data for similar tests with 6-aminonicotinamide are presented in Table 5. The presence of glycerophosphate in combination with 6-aminonicotinamide led to a significant rise in embryo mortality. Among embryos which survived the eighteenth day of incubation the incidence of micromelia and of abnormal formation of the beak was much reduced. It can be shown that as far as micromelia is concerned, which was quite obviously in these tests the principal effect of 6-aminonicotinamide, the lowering of incidence due to the presence of

glycerophosphate remains highly significant ($P < 0{\cdot}0001$) even on the unwarranted assumption that all undiagnosed embryos dying in the unsupplemented group had been normal and all those dying in the supplemented group had been abnormal! The same holds for frequency of over-all abnormality (or

Table 4. *The effect of treatment at 96 h of incubation with 0·6 mg/egg (4·95 μM) 3-acetylpyridine either alone or in combination with 10 mg/egg (30·86 μM)* DL-*α-glycerophosphate hexahydrate disodium salt*

(Supplement and teratogen injected together in the same vehicle.)

	3-Acetylpyridine	3-Acetylpyridine supplemented with glycerophosphate
Treated ...	236	229
Mortality to 18 days (%)	44·5	19·7
Survivors	133	183
Normal appearance (%)	15·8	80·3
Muscular hypoplasia (%)	82·0	19·1
Short upper beak (%)	13·5	0·5
Crooked neck (%)	8·3	0

Table 5. *The effect of treatment at 96 h of incubation with 5 γ/egg (0·035 μM) 6-aminonicotinamide either alone or in combination with 10 or 15 mg/egg (30·86 or 46·29 μM)* DL-*α-glycerophosphate hexahydrate disodium salt*

(Supplement and teratogen injected in the same vehicle.)

	6-Aminonicotinamide	6-Aminonicotinamide supplemented with glycerophosphate
Treated ...	251	235
Mortality to 18 days (%)	9·6	19·6
Survivors	227	189
Normal appearance (%)	11·9	41·8
Micromelia (%)	88·1	58·2
Abnormal beak (%)	27·8	14·8
Syndactylism	4·8	6·9
Miscellaneous defects (%)	1·3	0·5

of grossly normal appearance) in the two groups. Syndactylism occurred with slightly higher incidence in the presence of glycerophosphate, but the frequencies were low and the differences not statistically significant.

Our data demonstrate clearly that in the presence of glycerophosphate the teratogenic effects of the two niacin antagonists 3-acetylpyridine and 6-aminonicotinamide are greatly reduced. In contrast, no such beneficial results were

observed when, in work not reported in detail, glycerophosphate was used as supplement to treatment with either sulfanilamide (1·3 mg/egg), acetazolamide (13 mg/egg) or insulin (8 i.u.).

Tests with sodium ascorbate or ascorbic acid

The effects of sodium ascorbate or ascorbic acid as supplement to treatment with 3-acetylpyridine, 6-aminonicotinamide and sulfanilamide are illustrated by the data in Tables 6–10. With 3-acetylpyridine we conducted two tests. In the

Table 6. *The results of treatment at 96 h of incubation with 0·5 mg/egg (4·13 μM) 3-acetylpyridine either alone or combined with 10 mg/egg (50·47 μM) sodium ascorbate, buffered with ascorbic acid*

(Supplement and teratogen injected in the same vehicle.)

	3-Acetylpyridine	3-Acetylpyridine supplemented with ascorbate
Treated ...	75	67
Mortality to 18 days (%)	24·0	12·0
Survivors	57	59
Normal appearance (%)	43·9	88·1
Muscular hypoplasia (%)	56·1	10·2
Short upper beak (%)	5·3	1·7
Abnormal neck (%)	1·8	0
Miscellaneous defects (%)	0	1·7

Table 7. *The results of treatment at 96 h of incubation with 0·5 mg/egg (4·13 μM) 3-acetylpyridine either alone or combined with 10 mg/egg (56·78 μM) ascorbic acid*

(Supplement and teratogen injected together in propylene glycol.)

	3-Acetylpyridine	3-Acetylpyridine supplemented with ascorbic acid
Treated ...	163	151
Mortality to 18 days (%)	13·5	15·9
Survivors	141	127
Normal appearance (%)	79·4	98·4
Muscular hypoplasia (%)	19·8	1·6
Miscellaneous defects (%)	0·7	0

first of these the embryos were exposed either to an aqueous solution of 3-acetylpyridine (0·5 mg/egg) or to the same amount of the teratogen in an aqueous solution of sodium ascorbate (10 mg/egg), buffered with ascorbic acid (Table 6). In the presence of ascorbate embryo mortality was reduced and a much higher proportion of embryos escaped teratogenic effects, the incidence of muscular

hypoplasia and of shortening of the upper beak being greatly reduced. The differences in frequency of normal embryos and of specimens with muscular hypoplasia were highly significant between the two groups ($P \ll 0{\cdot}0001$). In the second test we used propylene glycol as solvent either for 3-acetylpyridine

Table 8. *The effects of treatment at 96 h of incubation with 5 γ/egg (0·035 μM) 6-aminonicotinamide either alone or supplemented with 10 mg/egg (50·47 μM) sodium ascorbate, buffered with ascorbic acid*

(Supplement and teratogen injected together in the same vehicle.)

	6-Aminonicotinamide	6-Aminonicotinamide supplemented with ascorbate
Treated ...	167	165
Mortality to 18 days (%)	14·4	10·3
Survivors	143	150
Normal appearance (%)	7·0	35·3
Micromelia (%)	92·3	64·0
Abnormal beak (%)	65·7	23·3
Syndactylism (%)	22·4	11·4
Miscellaneous defects (%)	0	0·7

Table 9. *The effects of treatment at 96 h of incubation with 1·3 mg/egg (7·55 μM) sulfanilamide either alone or supplemented with 10 mg/egg (50·47 μM) sodium ascorbate, buffered with ascorbic acid*

(Sulfanilamide injected in propylene glycol, followed by aqueous solution of ascorbate.)

	Sulfanilamide	Sulfanilamide supplemented with ascorbate
Treated ...	159	158
Mortality to 18 days (%)	22·6	29·1
Survivors	123	114
Normal appearance (%)	55·3	84·2
Micromelia (%)	43·9	15·8
Abnormal beak (%)	26·0	10·5
Miscellaneous defects (%)	0·8	0

alone or for its combination with ascorbic acid (Table 7). In propylene glycol as vehicle 3-acetylpyridine was less toxic and much less teratogenic than in aqueous solution. The incidence of muscular hypoplasia was greatly reduced and we did not find any cases of shortened upper beak or abnormality of the neck. With ascorbic acid as supplement the frequency of grossly normal embryos was much increased, with a corresponding drop in occurrence of muscular hypoplasia ($P \ll 0{\cdot}0001$).

Our results with 6-aminonicotinamide are presented in Table 8. Supplementing 6-aminonicotinamide with sodium ascorbate reduced embryo mortality to a statistically non-significant extent, definitely raised the incidence of grossly normal-appearing embryos and greatly lowered the occurrence of micromelia, beak defects and syndactylism. All these differences in teratogenic activity were statistically highly significant ($P \ll 0{\cdot}0001$).

Table 10. *The effect of treatment at 96 h of incubation with 1 mg/egg (5·81 μM) sulfanilamide either alone or supplemented with 10 mg/egg (56·78 μM) ascorbic acid*

(Supplement and teratogen injected together in propylene glycol.)

	Sulfanilamide	Sulfanilamide supplemented with ascorbic acid
Treated ...	153	159
Mortality to 18 days (%)	13·1	23·3
Survivors	133	122
Normal appearance (%)	63·2	90·2
Micromelia (%)	35·3	9·8
Abnormal beak (%)	18·0	6·6
Miscellaneous defects (%)	0·8	0

We made two experiments on the effect which supplementation with sodium ascorbate or ascorbic acid has on the teratogenicity of sulfanilamide. In the first of these tests (Table 9) we wished to compare the effect on embryos of 1·3 mg/egg sulfanilamide dissolved in propylene glycol with or without the subsequent additional injection of buffered sodium ascorbate. The results show that the presence of ascorbate increased embryo mortality slightly, but not significantly; the differences, on the other hand, of embryos which were grossly normal and of those with micromelia and beak defects were proof of a highly significant beneficial effect of supplementation with ascorbate, with probabilities of $\ll 0{\cdot}0001$ for frequencies of normal and micromelic embryos and of 0·01 for defects of the beak. In a second test we reduced slightly the amount of injected sulfanilamide and observed the effects alone or with the addition of ascorbic acid rather than sodium ascorbate (Table 10). Embryo mortality was somewhat increased in the presence of ascorbic acid over that following sulfanilamide alone (P 0·02); however, the incidence of normality and micromelia ($P \ll 0{\cdot}0001$) and of malformations of the beak ($P < 0{\cdot}01$) demonstrate that ascorbic acid and sodium ascorbate are similar in antiteratogenic activity.

In contrast to the beneficial effect which ascorbate has on the teratogenic activity of 3-acetylpyridine, 6-aminonicotinamide and sulfanilamide, we found that the combination of ascorbate with either acetazolamide or insulin was quite detrimental in its results on survival and development. This is illustrated

by the results of supplementing insulin with sodium ascorbate (Table 11). The data of this table do not, in fact, illustrate the full extent to which the insulin-induced aberrations of development were exaggerated, the defectiveness of the skeleton of limbs and beak being quantitatively much more extreme in the ascorbate-supplemented group than in that treated with insulin alone. The combination of acetazolamide and ascorbate led to similar potentiation.

Table 11. *The effect of treatment at 96 h of incubation with 4 units insulin either alone or supplemented with 10 mg/egg (56·78 μM) sodium ascorbate, buffered with ascorbic acid*

(Injection of insulin followed by aqueous solution of ascorbate.)

	Insulin	Insulin supplemented with ascorbate
Treated...	162	161
Mortality to 18 days (%)	33·3	62·7
Survivors	108	60
Normal appearance (%)	71·3	25·0
Micromelia (%)	24·1	73·3
Short upper beak (%)	13·0	50·0

A few remarks should finally be added which apply to all reported data. (1) Preliminary tests made earlier and control experiments done now have shown that neither succinate, nor glycerophosphate, nor sodium ascorbate or ascorbic acid when injected separately at 96 h of incubation, and at the dosages used for supplementation, produce toxic or teratogenic effects in chicken embryos. (2) In the tests in which teratogens were used either with or without metabolic supplements, we found that embryo mortality and teratogenicity were often, but by no means regularly, correlated. (3) All three types of supplements used in our tests, i.e. succinate, glycerophosphate and ascorbate, when effective at all, led to quantitatively similar beneficial results in all parts affected by the teratogens (general body growth, musculature, facial or appendicular skeleton). (4) The effects of succinate, glycerophosphate and ascorbate were limited to the dosages recorded in the text and to treatment at 96 h of incubation. Other dosages and stages of development might well have produced even more favorable results.

DISCUSSION

We shall open the discussion of our results with a quotation from the review on 'Biological oxidoreductions' by Ernster & Lee (1964): 'It is now firmly established that mitochondria and submitochondrial particles from a variety of tissues catalyse an energy-dependent reversal of electron transport through the respiratory chain. The energy for the reaction can be supplied either by

added ATP or by endogenous high-energy intermediates generated by the respiratory chain. In the former case, the reaction involves an actual reversal of oxidative phosphorylation, and there is now evidence that electron transport through all carriers of the respiratory chain, from NAD^+ to cytochrome oxidase, can be reversed in this way. When endogenous high-energy intermediates generated by the respiratory chain serve as the source of energy, the reversal of electron transport can proceed directly at the expense of the intermediates, without the involvement of the phosphorylating system. The two types of energy-dependent reversal of electron transport and their relation to oxidative phosphorylation are illustrated in Scheme 1' (Fig. 1). Ernster & Lee then show in their review that both types of energy-dependent reversal of electron transport in the respiratory chain have been studied by a number of investigators and under a great variety of conditions.

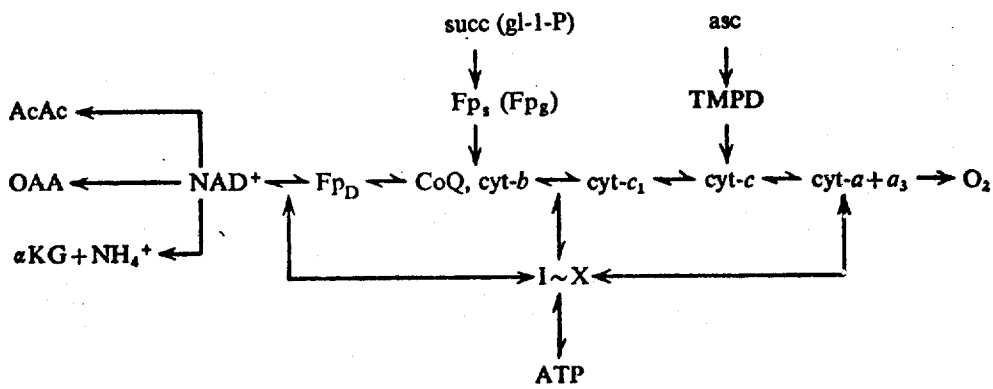

Fig. 1. Energy-dependent reversal of electron transport in the respiratory chain, and its relation to oxidative phosphorylation (courtesy Ernster & Lee, 'Biological oxidoreductions' and *Annual Reviews*, Inc., Palo Alto, California). AcAc, acetoacetate; asc, ascorbate; ATP, adenosine triphosphate; CoQ, coenzyme Q (ubiquinone); cyt, cytochrome; Fp_D, NADH dehydrogenase; FP_g, glycerol-1-phosphate dehydrogenase; Fp_S, succinate dehydrogenase; gl-1-P, glycerol-1-phosphate; I and X denote hypothetical energy-transfer carriers; αKG, α-ketoglutarate; NAD^+, nicotinamide adenine dinucleotide phosphate oxidized; OAA, oxaloacetate; succ, succinate; TMPD, *NNN'N'*-tetramethyl-*p*-phenylenediamine.

The high-energy intermediates shown in the scheme of Ernster & Lee are succinate, glycerophosphate and ascorbate. The importance of succinic acid and of glycerophosphic acid in processes of cellular oxidation had first been clearly enunciated by Meyerhof (1919). The mechanism of energy-dependent reversal of electron transport via succinate-linked reduction of NAD^+ and involving succinic dehydrogenase and the respiratory chain was well established by Chance & Hollunger (1957). Extensive documentation is also available for the conclusion that the glycerophosphate shuttle carries electrons from extra-mitochondrial DPNH to the intra-mitochondrial electron transport chain (Boxer & Devlin, 1961). The prominent role which ascorbic acid plays in elec-

tron transport was clearly brought out by Staudinger, Krisch & Leonhäuser (1961) and has since found ample verification.

The important basis for our present experimentation was the recognition that the high-energy intermediates succinate, glycerophosphate and ascorbate need not originate endogenously, but can play their role when derived from exogenous sources (review by Ernster & Lee, 1964; Ernster, 1965). This being so, it became our primary purpose to determine if these compounds can serve as exogenous supplements for relieving the noxious effects of specific teratogens when administered to developing chicken embryos. Our data show quite unambiguously that they can, indeed, be used in this manner. We could at the same time demonstrate clearly that their antiteratogenic effectiveness applies only to certain teratogens and not to others. This teratogen-specificity did not come as a surprise. Much of our earlier work had provided similar evidence, e.g. that succinate, far from being helpful, exaggerates insulin-induced effects in incidence and extent (Landauer & Rhodes, 1952) or that nicotinamide, which is so clearly antiteratogenic with insulin and many other teratogens, was found ineffective as supplement to either acetazolamide, dichlorphenamide or methazolamide, whereas ATP was helpful with either of the three (Landauer & Wakasugi, 1967, 1968). To these specific responses we shall return below.

It would seem well at this point to list compounds which in our experience have had alleviating properties in combination with teratogens that were used by us for treatment of chicken embryos after four days of incubation. They are the following: L-tryptophan hydrochloride (with 3-acetylpyridine and 6-aminonicotinamide, Landauer, 1957); α-ketoglutaric acid (with insulin, Landauer, 1948; Landauer & Rhodes, 1952); 3-hydroxyanthranilic acid (with 3-acetylpyridine and 6-aminonicotinamide, Landauer, 1957); pyruvate (with nicotine sulphate, Landauer, 1960*b*); nicotinamide (with insulin, Landauer, 1948; Landauer & Rhodes, 1952; with eserine, Landauer 1949; with sulfanilamide, Zwilling & DeBell, 1950; with pilocarpine, Landauer, 1953*b*; with 3-acetylpyridine and 6-aminonicotinamide, Landauer, 1957); ADP (with acetazolamide, dichlorphenamide and methazolamide, Landauer & Wakasugi, 1967, 1968); glucose (with pilocarpine, Landauer, 1956; with nicotine sulphate, Landauer, 1960*b*; with thallium acetate, Landauer, 1960*a*); riboflavin and riboflavin-5-phosphate (with boric acid, Landauer, 1952; Landauer & Clark, 1964). The metabolic functions which all of these compounds, used as supplements, have in common lie in their value for the production of cellular energy via phosphorylation and hydrogen transport (see Landauer & Wakasugi, 1968). It seems likely that one can add sorbitol (used with boric acid, Landauer, 1953*a*; with pilocarpine, Landauer, 1956; with thallium acetate, Landauer, 1960*a*), as well as sorbose, arabitol, imidazole and histidine (with thallium acetate, Landauer, 1960*a*), to this list since strong evidence has been presented by Boyer and his associates (Boyer, 1963; Boyer *et al.* 1963) in support of the conclusion that imidazole and phosphohistidine participate as intermediates in oxidative phos-

phorylation and since the same may well be true for the other polyhydroxy compounds used in our experiments. It is not surprising, therefore, that succinate, glycerophosphate and ascorbate should fall in line with the foregoing compounds in having antiteratogenic value.

Considerable evidence exists for the conclusion that interference with mitochondrial functions and with the activity of other cytoplasmic organelles is a major cause of teratogenesis. Conclusions to that effect were first arrived at by Braun (1954, 1964, 1966) on the basis of his work with Janus green B as it affected chicken embryos. Additional information has come from investigations of Kaplan & Johnson (1968), Aksu, Mackler, Shepard & Lemire (1968), Pollard & Fraser (1968), Verrusio, Pollard & Fraser (1968), Verrusio & Watkins (1969) and Chepenik, Johnson & Kaplan (1969). Details need not be discussed here. An interesting exposition of the basic problems was presented by Herken (1966).

Conclusive evidence for the importance of cellular energy in the origin of malformations can also be found in the role which general body growth plays in aberrations from normal morphogenesis. Such information is available from studies on hypoxia (Büchner, 1948, 1955; Rübsaamen, 1952; Degenhardt, 1954; Grabowski, 1970; and others), on hypocaloric diets (Runner & Miller, 1956; Kalter, 1960; Miller, 1962; Yasuda, Nanjo & Suzuki, 1966; Rogóyski, 1967; and others) and, of course, on the effects of antimetabolites. All students of experimental teratology are aware of the important relationship existing between general body growth and the occurrence of specific defects. Some metric data on dose and stage dependence are available for insulin and pilocarpine (Landauer, 1953*c*). Evidence suggesting that antimetabolites or other chemical teratogens interfere with growth rather than with differentiation became particularly impressive when it was found that repair of teratogenic damage with supplements of nicotinamide (or either its precursors or derivatives) was still possible an appreciable time *after* treatment with particular teratogens such as sulfanilamide (Zwilling & DeBell, 1950) or bidrin (Roger, 1967; Roger, Upshall & Casida, 1969).

The metabolic intermediates used in our present experiments, namely succinate, glycerophosphate and ascorbate, as well as the compounds which in our earlier work had been found to have alleviating or antiteratogenic properties, clearly have much in common as far as our interests are concerned. They also differ, however, in important ways. There is first of all the fact that in combination with the particular teratogens with which we tested them, only some of the supplements were helpful, while others were inert or even produced an exaggeration of teratogenic activity. It has been pointed out, for instance, that, used as supplement, ascorbate greatly reduced the teratogenic effects of 3-acetylpyridine, 6-aminonicotinamide and sulfanilamide, but strikingly potentiated incidence and gross expression of insulin-induced malformations, whereas nicotinamide, on the other hand, abolished or reduced the teratogenic activity of all these

compounds. It is clear, therefore, that there are separate ways by which teratogens interfere with energy production and by which the effects of teratogens can be mitigated. The details of such differences remain unknown.

Secondly, it seems clear from the results of our present experiments that teratogenic damage may occur at different points along one and the same pathway. For, our results have shown that in all instances in which succinate reduced the noxious effects of a teratogen, ascorbate had a quantitatively similar effect. This is of great interest since it is known that these two intermediates, whether contributed by endogenous or introduced from exogenous sources, become active at different sites of the respiratory chain. It seems very unlikely that three different teratogens (namely 3-acetylpyridine, 6-aminonicotinamide and sulfanilamide) should similarly impede the metabolic events occurring at both sites. Whatever their specific interference with mitochondrial activity, our results seem to demonstrate clearly that the role of the intermediates, when used as supplement to the teratogens, is that of stimulus for cellular energy production, thereby increasing the chances for protection of the embryo against teratogen-induced damage or of its repair.

RÉSUMÉ

Succinate, glycerophosphate et ascorbate, sources d'énergie cellulaire et substances antitératogènes

Nos recherches expérimentales montrent que le succinate et l'ascorbate réduisent considérablement les effets tératogènes de la 3-acetylpyridine, la 6-aminonicotinamide et la sulfanilamide. Le glycerophosphate a donné des résultats similaires lorsqu'il a été utilisé en association avec la 3-acetylpyridine et la 6-aminonicotinamide, mais non avec la sulfanilamide. Avec certains autres agents tératogènes ces intermédiaires à potentiel énergétique élevé n'ont pas manifesté d'action protectrice; dans quelques cas (acetazolamide, insuline) ils ont même eu pour résultat de renforcer les perturbations induites par les agents tératogènes.

Les résultats de nos expériences montent clairement que des intermédiaries à potentiel énergétique élevé, introduits dans le chaine respides des mitochondries, peuvent diminuer l'incidence et le degré d'expression des malformations produites par des agents tératogènes spécifiques. En accord avec des informations antérieures sur la nature des composés antitératogènes, on peut conclure que les substances tératogènes en question agissent par interférence avec la production d'énergie mitochondriale dans les tissus pour lesquels elles ont une affinité spécifique.

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EFFECT OF GLYCERINE AND GLYCEROPHOSPHATE ON CHOLESTEROL LEVEL IN THE BLOOD AND TISSUES UNDER CONDITIONS OF EXPERIMENTAL ATHEROSCLEROSIS

A. D. Liberman

At the present time a great amount of attention is being paid to questions of experimental atherosclerosis on the part of investigators in different specialties.

In recent years significant progress has been made in the study of cholesterol level under conditions of experimental atherosclerosis. However the mechanism of its disruption is still entirely unknown, although a large number of researchers have devoted themselves to this question.

Popjoak (3) showed that cholesterol feeding leads to the consumption of stored fat, to increased synthesis of plasma phospholipids, and to increased utilization of fatty acids. This points to the role of cholesterol as a regulator of lipid content in blood plasma. On the other hand, the initial increase in cholesterol is quantitatively proportional to the degree of the phospholipid increase. An initial triglyceride increase in plasma evokes not only an increase in cholesterol but in phospholipids as well (5). Thus, there is a not-fully-explained influence of these constituent parts of the plasma on each other.

If the effect of the fattiest acids on the development of atherosclerosis has been subjected to detailed study, the role of glycerine in this question has been studied very little.

Thus, V. N. Orekhovich and N. Ye. Plotnikova (1) found that after two weeks of feeding rabbits glycerine, the blood cholesterol increase was 100%. In a rabbit which was used in a 2-month experiment (with some interruptions), ulcers formed in the aorta wall, lower in the ribs, and in parts of the abdomen. In contrast to their data, persistent blood cholesterol and erythrocyte changes were not noted (6) in rabbits and guinea pigs which received gylcerine doses of 5-10 ml in a 50% solution over 30-40 days.

N. V. Sidorenkov and T. V. Babina (2), in studying the character of experimental hypercholesterinemia in a standard and during glycerine and glycerophosphate administration (50 mg/kg body weight), found that the introduction of glycerine and calcium glycerophosphate, in particular, increases hypercholesterinemia due to ester formation of cholesterol, which has hardly any effect on free cholesterol. We discovered no other data on the effect of glycerine and glycerophosphate. Considering what is given above, we set out for ourselves the problem of explaining the role of these substances in the mechanism of origin and development of experimental atherosclerosis.

DATA AND METHOD

The experiments were conducted on non-strain rabbits weighing 2.5-3.0 kg under identical conditions of maintenance and feeding. Experimental atherosclerosis was induced by feeding pure cholesterol in 0.5 g/ kg doses daily over a period of 4-4.5 months, mixed with root crops (carrots, beets). The following series of experiments were carried out.

1. Feeding the rabbits cholesterol over a 4-4.5 month period.
2. Feeding the rabbits cholesterol with the addition of small doses of glycerine (50 mg/kg body weight).
3. Feeding the rabbits cholesterol with the addition of small doses of calcium glycerophosphate (50 mg/kg).

The cholesterol level was determined by the digitonin method based on the Dieberman-Burckhardt color reaction.

The blood was studied for the development of experimental atherosclerosis 2, 3, and 4 months after the beginning of cholesterol feeding. Cholesterol was found in the blood, as well as in the liver, spleen, kidneys, adrenal gland, intestines, heart muscle, lungs, aorta, brain, and thigh muscle.

RESEARCH RESULTS AND CONCLUSIONS

First the cholesterol level was determined in blood and tissues in a standard and under experimental atherosclerosis evoked by cholesterol administration. According to the development of atherosclerosis, the general cholesterol level in the blood increased over two months to 345.7 mg %, over three months to 688 mg %, and then fell somewhat to 602 mg % over four months. Due to this, the increase in cholesterol occurs mainly because of ester-forming cholesterol, since free cholesterol increased by only a minor amount. The increase in cholesterol content is noted in all the organs and tissues, mainly due to esters of cholesterol (Table 1).

TABLE 1. CHOLESTEROL CONTENT IN BLOOD AND TISSUES IN RABBITS WITH EXPERIMENTAL ATHEROSCLEROSIS (MEAN DATA)

# of Exper.	Cholesterol content, in mg %					
	Experimental			Control		
	General	Esters	Free	General	Esters	Free
7	**Liver**					
	2043±354 +452% P=0,0012	1646±268 +877% P=0,001	397±127 +97% P=0,08	370±22,4	168,5±24	201,5±9
	Heart					
	285,7±22,4 +72,8% P=0,0019	190,7±13,1 +118% P=0,0012	95±12,3 +22,1% P=0,121	165,4±32,2	87,5±16,4	77,8±13,2
	Spleen					
	1175±214 +280,7% P=0,06	680±112,5 +480% P=0,0012	495±105,6 +158,7 P=0,014	308,6±38	117,3±40	191,3±35
	Kidney					
	511,4±56,6 +65,7% P=0,0019	212±23,4 +231,6% P=0,0009	299,4±10,8 +19,3% P=0,093	314,7±34,3	63,9±7	250,8±34,7
	Brain					
	2044±60,6 +39% P=0,0014	267±20,5 +31,5% P=0,13	1777±78 +40,2% P=0,0010	1470±135	203±71,8	1267±117,5
	Lungs					
	670±47 +90% P=0,0011	318±37,3 +191,7% P=0,0011	352±28 +47,8% P=0,0018	352,5±36	109±10	243,5±17,2
	Thigh muscle					
	134±21,5 +90,6% P=0,0021	73±10,8 +107% P=0,0018	61±11,9 +74,3% P=0,06	70,3±8,8	35,3±4	35±5,6
	Intestine					
	405±36,7 +100% P=0,0011	200±19,5 +177,4% P=0,0009	205±21,1 +57,2% P=0,0018	202,5±9,5	72,1±4	130,4±10
	Aorta					
	430±85,1 +115% P=0,014	232±42,9 +65,7% P=0,057	198±43,6 +230% P=0,0019	200±17,3	140±10,8	60±10,4
	Blood					
	610±102 +881% P=0,0011	488±95,6 +1151% P=0,0012	122±19,6 +439,4% P=0,0012	62±5,1	39±3,1	23±3,1
	Adrenal gland *					
	19,3±0,599 52% P=0,0018	17,68±1,058 +99,3% P=0,0014	1,62±0,17 −42,8% P=0,08	12,7±1,83	9,87±1,58	2,828±1,087

* Cholesterol content given in percent.

The cholesterol content increases especially in such organs as the liver, spleen, kidney, and adrenal gland, and in the blood.

With cholesterol feeding of rabbits along with the addition of small doses of glycerine (50 mg/kg weight), the character of change in general blood cholesterol level, with respect to atherosclerotic development, was the same as with pure cholesterol atherosclerosis, but its level was higher. If during feeding with cholesterol alone the general blood cholesterol level was 698 mg %, it equalled 979 mg % with the administration of a glycerine feeding, and with the appearance of morphological changes, these values equalled 602 and 755 mg % respectively. An increase in blood cholesterol (of 11.1 times) is noted in all the organs studied: in the liver (4.5 times), spleen (2.5 times), aorta (5.7 times), kidney (102%), adrenal gland (82.2%), intestines (88.1%), heart (70%), and lungs (64%) (Table 2).

TABLE 2. CHOLESTEROL LEVEL IN BLOOD AND TISSUES OF RABBITS WITH EXPERIMENTAL ATHEROSCLEROSIS WITH GLYCERINE ADDITION (MEAN DATA)

# of Exper.	Cholesterol content, mg %					
	Experimental				Control		
	General	Esters	Free		General	Esters	Free
7	2051±214 +454,3% P=0,0007	1737±234 +930,8% P=0,0008	314±29,8 -55,8% P=0,0021	Liver	370±22,4	163,5±24	201,5±9
7	281±6,8 +70% P=0,0017	205±18,3 +131% P=0,001	76±13 --2,4% --	Heart	165,3±32,2	87,5±16,4	77,8±13,2
7	1086±74,7 +252% P=0,0006	695±72 +500% P=0,0008	391±36,3 +104,3% P=0,0015	Spleen	308,6±38	117,3±40	191,3±36
7	637±69 +102,4% P=0,0013	307±46 +380,4% P=0,0011	330±30,2 +31,1% P=0,07	Kidney	314,7±34,3	63,0±7	250,8±34,7
7	2003±43,1 +36,2% P=0,0016	307±39,8 +51,2% P=0,093	1696±56,8 +33,8% P=0,014	Brain	1470±135	203±71,8	1267±147,5
7	578±43 +64% P=0,0018	300±35,3 +175,2% P=0,0011	278±38,2 +14,1% P=0,15	Lungs	352,5±36	109±10	243,5±17,2
7	109±10,7 +55% P=0,0021	60±5,6 +70% P=0,0016	49±10,7 +40% P=0,11	Thigh muscle	70,3±8,8	35,3±4	35±6,6
7	381±28,2 +88,1% P=0,00099	196±7,6 +171,8% P=0,0004	185±23,4 +41,9% P=0,054	Intestines	202,5±9,5	72,1±4	130,4±10
6	1350±364 +575% P=0,002	1037±211 +640,7% P 0,001	313±111 +421,7% P=0,019	Aorta	200±17,3	140±10,8	60±10,4
7	755±38,5 +1119% P=0,0003	549±19,4 +1307,7% P=0,0002	206±34,4 +800% P=0,0011	Blood	62±5,1	39±3,1	23±3,1
7	23,28±1,58 +83,2% P=0,0014	20,19±1,8 +104,5% P=0,00099	3,09±1,31 +9,2% P=0,6	Adrenal gland *	12,7±1,83	9,87±1,58	2,82±1,08

* Cholesterol content given in percentages.

During feeding of cholesterol to rabbits with the addition of glycerophosphate (50 mg/kg body weight), hypercholesterinemia set in earlier and was more noticeable. In two months cholesterol level equalled 807.4 mg %, in three months 803 mg %, and in four months 481.4 mg %.

Cholesterol content in tissues is presented in Table 3.

TABLE 3. CHOLESTEROL LEVEL IN BLOOD AND TISSUES OF RABBITS WITH EXPERIMENTAL ATHEROSCLEROSIS WITH THE ADDITION OF GLYCEROPHOSPHATE (MEAN DATA)

# of Exper.	Cholesterol content, mg %					
	Experimental				Control		
	General	Esters	Free		General	Esters	Free
10	1826±185 +302% P≥0,0006	1484±191 +780,7% P≥0,0007	342±57 +69,7% P≥0,013	Liver	370±22,4	168,5±24	201,5±9
10	249±14,5 +50,6% P≥0,013	177±14 +102,3% P≥0,0011	72±15,5 −7,5% P≥0,37	Heart	165,3±32,2	87,5±16,4	77,8±13,2
10	674±63,5 +118,4% P≥0,001	445±63,3 +279,3% P≥0,0011	229±34,4 +19,6% P≥0,14	Spleen	308,6±38	117,3±40	191,3±36
10	461±42,7 +47,4% P≥0,012	202±24,9 +216,1% P≥0,0009	262±34,3 +4,4% P≥0,56	Kidney	314,7±34,3	63,9±7	250,8±34,7
10	1810±52,6 +23,1% P≥0,15	310±17,7 +52,7% P≥0,08	1500±50,6 +18,4% P≥0,075	Brain	1470±135	203±71,6	1267±147,5
10	475±57,7 +31,7% P≥0,06	220±22,9 +101,8% P≥0,0011	255±41,2 +5% P≥0,37	Lungs	352±36	109±10	243,5±17,5
10	121,8±19,6 +73,2% P 0,013	83,2±14 +133,7% P≥0,0014	38,6±10,2 +10,3% P≥0,37	Thigh muscle	70,3±8,8	35,3±3	35±6,6
10	408,5±20,5 +101,7% P≥0,0005	187±10 +160% P≥0,0008	221,5±18 +70% P≥0,0011	Intestines	202,5±9,5	72,1±4	130,4±10
9	2302±637 +1051% P≥0,0015	1686,6±519 +1104,7% P 0,0015	615,4±39 +925,6% P≥0,064	Aorta	200±17,3	140±10,8	60±10,4
10	481,4±61,3 +676,4% P≥0,0007	366,8±55,3 +897% P≥0,0008	114,6±18,2 +402,5% P≥0,0009	Blood	62±5,1	39±3,1	23±3,1
10	17,16±0,73 +35,1% P≥0,051	15,81±0,754 +60,1% P≥0,0014	1,35±0,197 −52,3% P≥0,09	Adrenal gland *	12,7±1,83	0,87±1,58	2,83±1,08

*Cholesterol content given in percentages.

TABLE 4. CHOLESTEROL CONTENT IN CERTAIN ORGANS WITH PURE CHOLESTEROL ATHEROSCLEROSIS AND ATHEROSCLEROSIS WITH THE ADDITION OF GLYCERINE (MEAN DATA)

Cholesterol content in mg %							
Experimental				Control			
General	Esters	Free		General	Esters	Free
1350±364 +214% P≥0,016	1037±211 +347% P≥0,0016	313±111 +58% P≥0,121	Aorta	430±85,1	232±42,9	198±43,6
755±38,5 +23,7% P≥0,093	549±19,4 +12,5% P 0,2	206±31,4 +6% P≥0,057	Blood	610±102	488±95,6	122±19,6
637±69 +24,3% P≥0,086	307±46 +44,4% P≥0,067	330±30,2 +10,2% P≥0,067	Kidney	511,4±56,6	212±23,4	299,4±10,8
23,28±1,58 +20,6% P≥0,055	20,19±1,8 +11,2% P≥0,11	3,09±1,31 +91% P≥0,121	Adrenal gland *	19,3±0,799	17,68±1,58	1,62±0,47

* Cholesterol content given in percent.

Cholesterol content increased in the blood (6.7 times) and in all the organs, especially in the liver (3.9 times), 10.5 times in the aorta, spleen (1.1 times), intestines (101 %), heart (50.6 %), thigh muscle (73.2 %), and adrenal gland (35.1 %). In all the organs an increase in cholesterol occurs mainly due to ester-forming cholesterol.

Comparison of the amount of cholesterol accumulation in certain organs and tissues with pure cholesterol atherosclerosis (control) and atherosclerosis with the addition of glycerine (experimental) are presented in Table 4.

As is evident from the data presented in Table 4, an increase in general cholesterol content is noted in the aorta (214 %) and adrenal gland (20.6 %) compared with pure cholesterol atherosclerosis.

Concerning ester-forming cholesterol, its increase is noted in the kidney (44.4 %) and 3.47 times in the aorta. The free cholesterol content in the aorta increases 58 %, 69 % in the blood, and 91 % in the adrenal gland in comparison with pure cholesterol atherosclerosis. The changes in cholesterol content in other organs and tissues does not differ essentially.

TABLE 5. CHOLESTEROL CONTENT IN SEVERAL ORGANS WITH CHOLESTEROL ATHEROSCLEROSIS AND ATHEROSCLEROSIS WITH THE ADDITION OF GLYCEROPHOSPHATE TO CHOLESTEROL (MEAN DATA)

	Cholesterol content, mg %					
	Experimental (cholesterol + glycerophosphate)			Control (cholesterol)		
	General	Esters	Free	General	Esters	Free
Aorta	2302±637 +435,1% P=0,002	1696,6±169 +6,7% P=0,0018	615,4±30 +210% P=0,001	430±85,1	232±42,9	198±43,6
Blood	481,4±63,3 —21,2% P=0,121	366,8±55,3 —24,9% P=0,11	114,6±18,2 —6,1% P=0,43	610±102	488±95,6	122±19,6
Spleen	674±63,5 —42,7% P=0,055	445±63,3 —52,8% P 0,067	229±34,4 —46,2% P=0,015	1175±214	680±112,5	495±105,6
Lungs	475±51,7 —29,1% P=0,011	220±22,9 —44,5% P=0,055	255±41,2 —27,6% P=0,03	670±47	318±37,3	352±28

The relative cholesterol content data in several organs and tissues with pure cholesterol atherosclerosis (control) and atherosclerosis with the addition of glycerophosphate to cholesterol (experimental) are presented in Table 5.

The data presented in Table 5 indicate that, with the addition of glycerophosphate to cholesterol, an increase in all cholesterol fractions is observed in the aorta and a decrease in cholesterol in the spleen (general: 42.7 %, esters: 52.8 %, free: 116 %), lungs (general: 29.1 %, esters: 44.5 %, free: 27.6 %).

The changes in cholesterol content in the other organs and tissues did not essentially differ from each other.

Macroscopic atherosclerotic changes in organs with the addition of glycerophosphate to cholesterol are more intense, in our opinion.

The generalized data obtained by us indicate that glycerine addition to cholesterol increases subsequent atherogenic action in a number of

organs (adrenal gland, aorta).

The action of calcium glycerophosphate differs from the action of glycerine only in the distribution of cholesterol in rabbit organs and tissues. In the presence of more obvious expressions of atherosclerotic indicators, there is observed an increase in cholesterol only in the aorta. In other tissues (spleen, lungs) cholesterol is present in small amounts or remains unchanged in comparison with rabbit tissues in which atherosclerosis is evoked by giving cholesterol alone.

CONCLUSIONS

1. Addition of small doses of glycerine (50 mg/kg) to cholesterol feeding of rabbits leads to an increase in cholesterol content in the aorta and adrenal gland in comparison to tissues of rabbits which received only cholesterol.

2. Cholesterol content in the aorta of rabbits with experimental atherosclerosis increases under the influence of calcium glycerophosphate and decreases in the spleen and lungs, although pathomorphologic changes peculiar to experimental atherosclerosis are more obvious.

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ВЛИЯНИЕ ГЛИЦЕРИНА И ГЛИЦЕРОФОСФАТА НА ОБМЕН ХОЛЕСТЕРИНА В КРОВИ И ТКАНЯХ ПРИ ЭКСПЕРИМЕНТАЛЬНОМ АТЕРОСКЛЕРОЗЕ

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В настоящее время вопросам экспериментального атеросклероза уделяется очень большое внимание со стороны исследователей различных специальностей.

За последнее время достигнут значительный прогресс в изучении обмена холестерина при экспериментальном атеросклерозе. Однако все еще неизвестен механизм его нарушения, хотя этому вопросу посвящено большое число исследований.

Новак (3) показал, что скармливание холестерина приводит к расхолаживанию жира из депо, усиленному синтезу фосфолипидов плазмы и повышенному использованию жирных кислот. Это указывает на роль холестерина как регулятора состава липидов плазмы крови. С другой стороны, первичное увеличение содержания фосфолипидов (4) вызывает вторичное повышение холестерина, количественно пропорциональное степени повышения фосфолипидов. Первичное повышение триглицеридов в плазме вызывает повышение не только холестерина, но и фосфолипидов (5). Таким образом, существует не вполне выясненное влияние этих составных частей плазмы друг на друга.

Если влияние высших жирных кислот на развитие атеросклероза подвергалось тщательному изучению, то роль глицерина в этом вопросе изучалась очень мало.

Так, В. Н. Орехович и Н. Е. Плотникова (1) нашли у кроликов после 2 недель кормления глицерином увеличение холестерина в крови на 100%. У кролика, который находился в опыте около 2-х месяцев (с некоторым перерывом), в стенке аорты, но ниже дуги и в брюшной части, образовались язвы. В противоположность их данным не было отмечено (6) стойких изменений холестерина в плазме крови и эритроцитах у кроликов и морских свинок при приеме глицерина в доле 5—10 мл 50% раствора в течение 30—40 дней.

Таблица 1

Содержание холестерина в крови и тканях кроликов при экспериментальном холестериновом атеросклерозе (средние данные)

К-во опытов	Содержание холестерина в мг%					
	Опыт			Контроль		
	общий	эфиры	свободный	общий	эфиры	свободный
	Печень					
7	2043 ± 354 +452% P = 0,0012	1646 ± 268 +877% P = 0,001	397 ± 127 +97% P = 0,08	370 ± 22,4	168,5 ± 24	201,5 ± 9
	Сердце					
7	285,7 ± 22,4 +72,8% P = 0,0019	190,7 ± 13,1 +118% P = 0,0012	95 ± 12,3 +22,1% P = 0,121	165,4 ± 32,2	87,5 ± 16,4	77,8 ± 13,2
	Селезенка					
7	1175 ± 214 +280,7% P = 0,06	680 ± 112,5 +483% P = 0,0012	495 ± 105,6 +158,7 P = 0,014	308,6 ± 38	117,3 ± 40	191,3 ± 36
	Почка					
7	511,4 ± 56,6 +65,7% P = 0,0019	212 ± 23,4 +231,6% P = 0,0009	299,4 ± 10,8 +19,3% P = 0,093	314,7 ± 34,3	63,9 ± 7	250,8 ± 34,7
	Головной мозг					
7	2044 ± 60,6 +37% P = 0,0014	267 ± 20,5 +31,5% P = 0,13	1777 ± 78 +40,2% P = 0,0010	1470 ± 135	203 ± 71,8	1267 ± 147,5
	Легкие					
7	670 ± 47 +90% P = 0,0011	318 ± 37,3 +191,7% P = 0,0011	352 ± 28 +47,8% P = 0,0018	352,5 ± 36	109 ± 10	243,5 ± 17,2
	Бедренная мышца					
7	134 ± 21,5 +90,6% P = 0,021	73 ± 10,8 +107% P = 0,0018	61 ± 11,9 +74,3% P = 0,06	70,3 ± 8,8	35,3 ± 4	35 ± 5,6
	Кишечник					
7	405 ± 36,7 +100% P = 0,0011	200 ± 19,5 +177,4% P = 0,0009	205 ± 21,1 +57,2% P = 0,0018	202,5 ± 9,5	72,1 ± 4	130,4 ± 10
	Аорта					
7	430 ± 85,1 +115% P = 0,014	232 ± 42,9 +65,7% P = 0,057	198 ± 43,6 +23,0% P = 0,0019	200 ± 17,3	140 ± 10,8	60 ± 10,4

К-во опытов	Содержание холестерина в мг%					
	Опыт			Контроль		
	общий	эфиры	свободный	общий	эфиры	свободный
	Кровь					
7	610 ± 102 + 884% P 0,0011	488 ± 95,6 + 1151% P 0,0012	122 ± 19,6 430,4% P 0,0012	62 ± 5,1	39 ± 3,1	23 ± 3,1
	Надпочечник*					
7	19,3 ± 0,799 52% P 0,0018	17,68 ± 1,058 99,3% P 0,0014	1,62 ± 0,47 + 42,8% P 0,08	12,7 ± 1,83	9,87 ± 1,58	2,828 ± 1,087

* Содержание холестерина дано в процентах.

Особенно увеличивается содержание холестерина в таких органах как печень, селезенка, почка, надпочечник и в крови.

При кормлении кроликов холестерином с добавлением малых доз глицерина (50 мг/кг веса) характер изменения общего холестерина крови, по мере развития атеросклероза, был таким же как и при чистом холестериновом атеросклерозе, но уровень его был выше. Если при кормлении одним холестерином содержание общего холестерина в крови было 698 мг%, то при прибавлении к пище глицерина он был равен 979 мг%, а при появлении морфологических изменений эти цифры соответственно равнялись 602 и 755 мг%. Отмечается увеличение содержания холестерина в крови (в 11,1 раза) и во всех исследованных органах: в печени (в 4,5 раза), селезенке (в 2,5 раза), аорте (в 5,7 раза), почке (на 102%), надпочечнике (на 82,2%), кишечнике (на 88,1%), сердце (на 70%), легких (на 64%), (таблица 2).

Таблица 2

Содержание холестерина в крови и тканях кроликов при экспериментальном холестериновом атеросклерозе с добавлением глицерина (средние данные)

К-во опытов	Содержание холестерина в мг%					
	Опыт			Контроль		
	общий	эфиры	свободный	общий	эфиры	свободный
	Печень					
7	2051 ± 214 + 454,3% P 0,0007	1737 ± 234 930,8% P 0,0008	314 ± 28,8 55,8% P 0,0021	370 ± 22,4	168,5 ± 24	201,5 ± 9
	Сердце					
7	281 ± 6,8 + 70% P 0,0017	205 ± 18,3 + 131% P 0,001	76 ± 13 + 2,1%	165,3 ± 32,2	87,5 ± 16,4	77,8 ± 13,2

К-во опытов	Содержание холестерина в мг%					
	Опыт			Контроль		
	общий	эфиры	свободный	общий	эфиры	свободный
	Селезенка					
7	1086 ± 74,7 + 252% P 0,0006	695 ± 72 + 500% P 0,0008	391 ± 36,3 + 104,3% P 0,0015	308,6 ± 38	117,3 ± 40	191,3 ± 36
	Почка					
7	637 ± 69 + 102,4% P 0,0013	307 ± 46 + 380,4% P 0,0011	330 ± 30,2 + 31,1% P 0,07	314,7 ± 34,3	63,9 ± 7	250,8 ± 34,7
	Головной мозг					
7	2003 ± 43,1 + 36,2% P 0,0016	307 ± 39,8 + 51,2% P 0,093	1696 ± 56,8 + 33,8% P 0,014	1470 ± 135	203 ± 71,8	1267 ± 147,5
	Легкие					
7	578 ± 43 + 64% P 0,0015	300 ± 35,3 + 175,2% P 0,0011	278 ± 38,2 + 14,1% P 0,15	352,5 ± 36	109 ± 10	243,5 ± 17,2
	Бедренная мышца					
7	109 ± 10,7 + 55% P 0,0021	60 ± 5,6 + 70% P 0,0016	49 ± 10,7 + 40% P 0,11	70,3 ± 8,8	35,3 ± 4	35 ± 6,6
	Кишечник					
7	381 ± 28,2 + 88,1% P 0,00099	196 ± 7,6 + 171,8% P 0,0004	185 ± 23,4 + 41,9% P 0,054	202,5 ± 9,5	72,1 ± 4	130,4 ± 10
	Аорта					
6	1350 ± 364 + 575% P 0,002	1037 ± 211 + 640,7% P 0,001	313 ± 111 + 421,7% P 0,019	200 ± 17,3	140 ± 10,8	60 ± 10,4
	Кровь					
7	755 ± 38,5 + 1119% P 0,0003	549 ± 19,4 + 1307,7% P 0,0002	206 ± 34,4 + 800% P 0,0011	62 ± 5,1	39 ± 3,1	23 ± 3,1
	Надпочечник*					
7	23,28 ± 1,58 + 83,2% P 0,0014	20,19 ± 1,8 + 104,5% P 0,00099	3,09 ± 1,31 + 9,2% P 0,6	12,7 ± 1,83	9,87 ± 1,58	2,82 ± 1,08

* Содержание холестерина дано в %%.

При кормлении кроликов холестерином с добавлением глицерофосфата (50 мг/кг веса тела) гиперхолестеринемия наступала

раньше и была более выражена. Через два месяца уровень холестерина равнялся 807,4 мг%, через 3 месяца 803 мг% и через 4 месяца — 481,4 мг%.

Содержание холестерина в тканях приведено в таблице 3.

Таблица 3

Содержание холестерина в крови и тканях кроликов при экспериментальном холестериновом атеросклерозе с добавлением глицерофосфата (средние данные)

К-во опытов	Содержание холестерина в мг%					
	Опыт			Контроль		
	общий	эфиры	свободный	общий	эфиры	свободный
	Печень					
10	1826 ± 185 + 3,2% P 0,0006	1484 ± 191 + 780,7% P 0,0007	342 ± 57 + 69,7% P 0,013	370 ± 22,4	168,5 ± 24	201,5 ± 9
	Сердце					
10	249 ± 14,5 + 50,6% P 0,013	177 ± 14 + 102,3% P 0,0011	72 ± 15,5 —7,5% P 0,37	165,3 ± 32,2	87,5 ± 16,4	77,8 ± 13,2
	Селезенка					
10	671 ± 63,5 + 118,4% P 0,001	435 ± 61,3 + 271,3% P 0,0011	2 9 ± 34,1 + 19,6% P 0,14	308,6 ± 38	117,3 ± 40	191,3 ± 36
	Почка					
10	464 ± 42,7 + 47,4% P 0,012	202 ± 24,9 +216,1% P=0,0009	262 ± 34,3 + 4,4% P=0,56	314,7 ± 34,3	63,9 ± 7	250,8 ± 34,7
	Головной мозг					
10	1810 ± 52,6 +23,1% P 0,15	310 ± 17,7 + 52,7% P 0,08	1500 ± 50,6 + 18,4% P=0,075	1470 ± 135	203 ± 71,8	1267 ± 147,5
	Легкие					
10	475 ± 57,7 + 31,7% P 0,06	220 ± 22,9 + 101,8% P 0,0011	255 ± 41,2 + 5% P 0,37	352 ± 36	109 ± 10	243,5 ± 17,5
	Бедренная мышца					
10	121,8 ± 19,6 + 73,2% P 0,013	83,2 ± 14 + 135,7% P 0,0014	38,6 ± 10,2 + 10,3% P 0,37	70,3 ± 8,8	35,3 ± 3	35 ± 6,6
	Аорта					
9	2392 ± 6 7 + 1051% P 0,0015	1686,6 ± 549 +1104,7% P 0,0015	615,4 ± 39 + 25,6% P=0,064	200 ± 17,3	140 ± 10,8	60 ± 10,4

К-во опытов	Содержание холестерина в мг%					
	Опыт			Контроль		
	общий	эфиры	свободный	общий	эфиры	свободный
	Кишечник					
10	408,5 ± 20,5 + 101,7% P 0,0005	187 ± 19 + 160% P 0,0008	221,5 ± 18 + 70% P 0,0011	202,5 ± 9,5	72,1 ± 4	130,4 ± 10
	Кровь					
10	481,4 ± 64,3 + 676,4% P 0,0007	366,8 ± 55,3 + 89,7% P 0,0008	114,6 ± 18,2 + 402,5% P 0,0009	62 ± 5,1	30 ± 3,1	23 ± 3,1
	Надпочечник*					
10	17,16 ± 0,73 + 35,1% P 0,051	15,81 ± 0,754 + 60,1% P 0,0014	1,35 ± 0,197 — 52,3% P 0,09	12,7 ± 1,83	9,87 ± 1,58	2,83 ± 1,08

*) Содержание холестерина дано в %%.

Таблица 4

Содержание холестерина в некоторых органах при чистом холестериновом атеросклерозе и атеросклерозе с добавлением глицерина (средние данные)

Содержание холестерина в мг%					
опыт (холестерин + глицерин)			контроль (холестерин)		
общий	эфиры	свободный	общий	эфиры	свободный
Аорта					
1350 ± 364 + 214% P 0,016	1037 ± 211 + 347% P 0,0016	313 ± 111 + 58% P 0,121	430 ± 85,1	232 ± 42,9	198 ± 43,6
Кровь					
755 ± 38,5 + 23,7% P 0,093	549 ± 19,4 + 12,5% P 0,2	206 ± 34,4 + 63% P 0,057	610 ± 102	488 ± 95,6	122 ± 19,6
Почка					
637 ± 69 + 24,3% P 0,086	307 ± 46 + 44,4% P 0,067	330 ± 30,2 + 10,2% P 0,067	511,4 ± 56,6	212 ± 23,4	299,4 ± 10,8
Надпочечник*					
23,28 ± 1,58 + 20,6% P 0,055	20,19 ± 1,8 + 11,2% P 0,11	3,09 ± 1,31 + 91% P 0,121	19,3 ± 0,799	17,68 ± 1,58	1,62 ± 0,47

* содержание холестерина дано в процентах.

Содержание холестерина увеличилось в крови (в 6,7 раза) и во всех органах, особенно в печени (в 3,9 раза), в 10,5 раз в аорте, селезенке (в 1,1 раза), кишечнике (на 101%), сердце (на 50,6%), бедренной мышце (на 73,2%), надпочечнике (на 35,1%). Во всех органах увеличение холестерина идет в основном за счет эфиросвязанного холестерина.

Сравнение величин накопления холестерина в некоторых органах и тканях при чистом холестериновом атеросклерозе (контроль) и атеросклерозе с добавлением глицерина (опыт) приведено в таблице 4.

Как видно из данных приведенных в таблице 4, отмечается увеличение содержания общего холестерина в аорте (на 214%) и надпочечнике (на 20,6%) по сравнению с чистым холестериновым атеросклерозом.

Что касается эфиросвязанного холестерина, то отмечается его увеличение в почке на 44,4% и в аорте в 3,17 раза. Содержание свободного холестерина увеличивается в аорте на 58%, крови на 69% и надпочечнике на 91% по сравнению с чистым холестериновым атеросклерозом. Изменения в содержании холестерина в других органах и тканях существенно не отличаются между собой.

Таблица 5

Содержание холестерина в некоторых органах при холестериновом атеросклерозе и атеросклерозе с добавлением к холестерину глицерофосфата (средние данные)

Содержание холестерина в мг%					
опыт (холестерин + глицерофосфат)			Контроль (холестерин)		
общий	эфиры	свободный	общий	эфиры	свободный
Аорта					
2302±637 +435,1% P 0,002	1686,6±469 +617% P 0,0018	615,4±30 +210% P 0,093	430±85,1	232±42,9	198±43,6
Кровь					
481,4±63,3 —21,2% P 0,121	366,8±55,3 —24,9% P 0,11	114,6±18,2 —6,1% P 0,43	610±102	488±95,6	122±19,6
Селезенка					
674±63,5 —42,7% P 0,055	445±63,3 —52,8% P 0,067	229±34,4 —46,2% P 0,015	1175±214	680±112,5	495±105,6
Легкие					
475±51,7 —29,1% P 0,014	220±22,9 —41,5% P 0,055	255±41,2 —27,6% P 0,63	670±47	318±37,3	352±28

Tr. Kuibyshevsk. Med. Inst. 29:46-50. 1964.

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TISSUE RESPIRATION RATE IN CERTAIN ORGANS IN RABBITS UNDER THE INFLUENCE OF LONG-TERM ADMINISTRATION OF GLYCERINE AND GLYCEROPHOSPHATE

L. V. Malysheva

The effect of glycerine on the activity of certain enzymes was studied by V. N. Orekhovich and N. Ye. Plotnikova (1) and N. Ye. Plotnikova (2). Its inhibitory action on protein-splitting enzymes - plasmin and trypsin - was established by the authors. However we found no research in the literature which was devoted to the study of the effect of glycerine and its product, glycerophosphate, on processes of tissue respiration.

In connection with the fact that a collection of co-workers at the Department of Biochemistry under the leadership of Prof. I. V. Sidorenkov are concerned with the study of the effect of glycerine and glycerophosphate on the exchange of matter in the living organism, we set out to study the action of these substances on tissue respiration rate. This study, in our point of view, is most necessary to the explanation of the mechanism of formation and development of experimental atherosclerosis.

METHODOLOGY

The studied were conducted on different species of rabbits of approximately the same age and weight, which received daily doses of 50 mg of glycerine or glycerophosphate / kg live weight over a period of five months. At the end of the period the animals were killed with an air embolism. Nine rabbits of the same age and weight served as a control under the same conditions, but they did not receive a stress factor.

Tissue respiration rate was determined by the manometric method in a Barburg apparatus, at a temperature of 37.5 deg in an oxygen atmosphere. Calculation was done in microliters of oxygen absorbed in one hour, based on 1 mg dried tissue weight (Q_{O_2}).

RESULTS OF THE STUDIES

In the first series of experiments the effect of long-term introduction of glycerine was studied in relation to brain tissue respiration rate. A paste made of 100 mg of the left hemisphere was selected for the experiment.

The results of the calculations are given in Table 1.

From the data presented in Table 1, it is evident that oxygen absorption by brain tissue in the control group was 3.25 μl on the average. Under the influence of administered glycerine, it decreased to 2.71 μl, by 16.7 %. With a statistical analysis, this difference proved to be significant ($p = 0.001$).

TABLE 1. RESPIRATION RATE IN RABBIT BRAINS UNDER THE INFLUENCE OF LONG-TERM GLYCERINE ADMINISTRATION

Oxygen absorption in μl/mg dried tissue wt, Q_{O_2}

	Control		Exper.
1	2,93		2,69
2	3,18		2,96
3	4,32		2,41
4	3,15		2,92
5	3,25		2,75
6	2,70		2,92
7	3,28		2,44
8	3,35		2,64
9	3,08		2,88
10	—		2,49
M	3,25		2,71
± m	0,15		0,06
P		0,001	

TABLE 2. TISSUE RESPIRATION RATE IN THE LIVER UNDER THE INFLUENCE OF LONG-TERM ADMINISTRATION OF GLYCERINE TO ANIMALS

Oxygen absorption in μl/mg dried tissue wt, Q_{O_2}

	Control		Exper.
1	1,74		0,69
2	1,30		0,99
3	2,31		1,16
4	1,97		1,21
5	1,94		1,36
6	1,34		1,13
7	1,42		1,28
8	2,31		1,26
9	2,43		1,04
10	—		1,11
M	1,86		1,12
±m	0,15		0,072
P	0,001		

The second organ which underwent study was the liver. In the experiment 200 mg of paste made of the left interior section was selected. The conditions of the experiments were analogous to the previous ones. The results obtained during the study of liver respiration rate under the influence of long-term administration of glycerine to rabbits are shown in Table 2.

The data given in Table 2 indicate that oxygen absorption in the liver tissue of the control animals was 1.86 μl on the average, while in the experimental rabbits it equalled 1.12 μl. Consequently, tissue respiration decreased 39.8 % in this case, which is significant statistically (p = 0.001).

For the study of heart respiration rate, pieces of muscle were taken from the left ventricle, a past of which, weighing 100 mg, was placed in a Barburg vessel. The results of the experiment and the data from a statistical analysis are presented in Table 3.

As the data presented in Table 3 indicate, oxygen absorption in the heart muscles from the control group equalled 2.16 μl on the average. Under the influence of long-term administration of glycerine, it decreased sharply, equalling 1.00 μl on the average. This decrease in respiration of 53.5 % was statistically reliable ($p < 0.001$).

TABLE 3. RESPIRATION RATE OF RABBIT HEART MUSCLE UNDER THE INFLUENCE OF LONG-TERM GLYCERINE ADMINISTRATION

Oxygen absorption in μl/mg dried tissue wt

	Control		Exper.
1	1,86		1,23
2	1,80		0,76
3	3,06		0,60
4	2,00		0,95
5	2,37		1,12
6	1,61		0,97
7	2,26		0,69
8	2,33		0,94
9	2,18		1,41
10			1,38
M	2,16		1,005
±m	0,14		0,088
P		0,0007	

TABLE 4. BRAIN RESPIRATION RATE UNDER THE LONG-TERM INFLUENCE OF CALCIUM GYLCEROPHOSPHATE ADMINISTRATION TO RABBITS

Oxygen absoprtion in μl/mg dried tissue wt.

	Control		Exper.
1	2,93		2,78
2	3,18		2,62
3	4,32		2,16
4	3,15		3,18
5	3,25		3,41
6	2,70		2,90
7	3,28		2,03
8	3,35		2,47
9	3,08		—
M	3,25		2,69
±m	0,15		0,17
P		0,01	

The experiments on the effect of glycerine thus indicate that, in administration in small doses over a long period of time, it essentially retards the process of tissue respiration in the brain, liver, and especially in the heart muscle.

For the study of the effect of giving calcium glycerophosphate to animals, we again began with brain tissue. The left hemisphere, as before, was used in the experiment. The experimental data are presented in Table 4.

The results of the experiment with the administration of calcium glycerophosphate which are presented in Table 4 indicate that in this case brain tissue respiration changed in relation to the control. If oxygen absorption in the control was equal to 3.25 μl on the average, then after long-term administration of calcium glycerophosphate, it was 2.69 μl on the average, a decrease of 17.2 %. This difference was statistically significant (p = 0.01).

The next organ studied was the liver. In Table 5 the results are presented for determinations of tissue resipration activity in the liver for a standard and after long-term administration of calcium glycerophosphate to rabbits.

TABLE 5. TISSUE RESPIRATION RATE IN THE LIVER UNDER THE INFLUENCE OF LONG-TERM ADMINISTRATION OF CALCIUM GLYCEROPHOSPHATE TO RABBITS

Oxygen absorption in μl/mg dried tissue wt.

	Control		Exper.
1	1,71		1,13
2	1,30		1,35
3	2,31		1,27
4	1,97		1,59
5	1,94		1,98
6	1,33		1,27
7	1,42		1,39
8	2,31		1,82
9	2,43		—
M	1,80		1,47
±m	0,15		0,104
P		0,05	

TABLE 6. TISSUE RESPIRATION RATE OF HEART MUSCLE UNDER THE INFLUENCE OF LONG-TERM ADMINISTRATION OF CALCIUM GLYCEROPHOSPHATE TO RABBITS

Oxygen absorption in μl/mg dried tissue wt.

	Control		Exper.
1	1,86		1,47
2	1,80		1,05
3	3,06		1,52
4	2,00		1,60
5	2,37		1,92
6	1,61		1,28
7	2,26		1,39
8	2,33		1,34
9	2,18		
M	2,16		1,45
±m	0,14		0,08
p		0,001	

As is evident from the data in Table 5, oxygen absoprtion by liver tissue was on the average of 1.45 μl in the experimental group, i.e. it decreased compared with the control by 21 %. In the statistical analysis this decrease proved to be significant: p = 0.05.

Finally, the last organ which we studied was the heart. The amount of oxygen absoprtion in heart muscle after long-term administration of calcium glycerophosphate to rabbits is given in Table 6.

On the basis of the experimental results given in Table 6, it is not difficult to see that a significant change in tissue respiration rate occurred in the heart muscle under long-term exposure to calcium glycerophosphate. Oxygen absoprtion decreased from a norm of 2.16 μl to 1.45 μl in the experimental rabbits. This amount of decrease (32.9 %) proved to be completely reliable upon statistical analysis (p = 0.001).

The generalized data which we obtained in the long-term exposure studies of rabbits given calcium glycerophosphate permit the conclusion that calcium glycerophosphate also lowers the rate of the most important process in the organism, the respiration rate. This decrease is, however, less obvious under the influence of free glycerine, a fact which is evidently associated with the presence of the glycerophosphate molecule of phosphoric acid, thanks to which it is more actively included in the exchange of matter and is more rapidly oxidized, than is free glycerine.

CONCLUSIONS

1. Daily administration to healthy rabbits of glycerine in doses of 50 mg/kg live weight over a period of five months leads to a decrease in tissue respiration in the brain (16.7 %, p = 0.001), liver (39.8 %, p = 0.001), and heart muscle (53.5 %, p < 0.001).

2. Administration to healthy rabbits in the same dosage and for the same length of time of calcium glycerophosphate as the product of glycerine in the organism, also leads to a decrease intissue respiration rate in the brain (17.2 %, p = 0.01), liver (21.0 %, p = 0.05), and heart muscle (32.9 %, p = 0.001).

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ИНТЕНСИВНОСТЬ ТКАНЕВОГО ДЫХАНИЯ НЕКОТОРЫХ ОРГАНОВ КРОЛИКОВ ПОД ВЛИЯНИЕМ ДЛИТЕЛЬНОГО ВВЕДЕНИЯ ГЛИЦЕРИНА И ГЛИЦЕРОФОСФАТА

Л. В. МАЛЫШЕВА

Влияние глицерина на активность некоторых ферментов исследовали В. Н. Орехович и Н. Е. Плотникова (1) и Н. Е. Плотникова (2). Авторами установлено его ингибиторное действие на протеолитические ферменты — плазмин и трипсин. Однако исследований, посвященных изучению влияния глицерина и его производного — глицерофосфата — на процессы тканевого дыхания, мы не обнаружили в литературе.

В связи с тем, что коллектив сотрудников кафедры биохимии под руководством профессора И. В. Сидоренкова занимается изучением влияния глицерина и глицерофосфата на обмен веществ в живом организме, мы поставили целью изучить действие этих веществ на интенсивность тканевого дыхания. Это исследование, с нашей точки зрения, крайне необходимо для выяснения механизма возникновения и развития экспериментального атеросклероза.

Методика

Исследования производили на разнопородных кроликах примерно одинакового возраста и веса, которые получали с пищей ежедневно в течение 5 месяцев глицерин или глицерофосфат в дозе 50 мг на 1 кг веса животного. В конце срока животных забивали воздушной эмболией. Контролем служили 9 кроликов того же возраста и веса, находившихся в тех же условиях, но не получавших нагрузки.

Интенсивность тканевого дыхания определяли манометрическим методом в аппарате Варбурга, при температуре 37,5°, в

атмосфере кислорода. Расчет производили в микролитрах кислорода, поглощенного за 1 час, отнесенных к 1 мг сухого веса ткани (Q_{O_2}).

Результаты исследований

В первой серии опытов было изучено влияние длительного введения глицерина на интенсивность тканевого дыхания головного мозга. В опыт брали 100 мг кашицы левого полушария головного мозга.

Результаты определений помещены в таблице 1.

Из данных, приведенных в таблице 1, видно, что поглощение кислорода тканью мозга в контроле составило в среднем 3,25 мкл. Под влиянием вводимого глицерина оно снизилось до 2,71 мкл, то-есть на 16,7%. При статистической обработке эта разница оказалась достоверной (p = 0,001).

Таблица 1

Интенсивность дыхания мозга кроликов под влиянием длительного введения глицерина

	Поглощение кислорода в мкл на 1 мг сухого веса ткани Q_{O_2}		
	контроль		опыт
1	2,93		2,69
2	3,18		2,96
3	4,32		2,41
4	3,15		2,92
5	3,25		2,75
6	2,70		2,92
7	3,28		2,44
8	3,35		2,64
9	3,08		2,88
10	—		2,49
M	3,25		2,71
± m	0,15		0,06
P		0,001	

Таблица 2

Интенсивность тканевого дыхания печени под влиянием длительного введения животным глицерина

	Поглощение кислорода в мкл на 1 мг сухого веса ткани		
	контроль		опыт
1	1,74		0,69
2	1,30		0,99
3	2,31		1,16
4	1,97		1,21
5	1,94		1,36
6	1,34		1,13
7	1,42		1,28
8	2,31		1,26
9	2,43		1,04
10	—		1,11
M	1,86		1,12
m	0,15		0,072
P	0,001		

Вторым органом, подлежащим исследованию, была печень. В опыт брали 200 мг кашицы левой внутренней доли ее. Условия опытов были аналогичны предыдущим. Результаты, полученные при изучении интенсивности дыхания печени под влиянием длительного введения кроликам глицерина, демонстрируются в таблице 2.

Данные, помещенные в таблице 2, показывают, что поглоще-

ние кислорода тканью печени у контрольных животных составило в среднем 1,86 мкл, тогда как у подопытных кроликов оно равнялось 1,12 мкл. Следовательно, тканевое дыхание снизилось в этом случае на 39,8%, что статистически является достоверным ($p = 0,001$).

Для изучения интенсивности тканевого дыхания сердца брали кусочек мышцы левого желудочка, кашицу которого весом в 100 мг погружали в сосудик Варбурга. Результаты исследования и данные статистической обработки приведены в таблице 3.

Как показывают данные, приведенные в таблице 3, поглощение кислорода мышцей сердца в контроле равно в среднем 2,16 мкл. Под влиянием же длительного введения глицерина оно резко снизилось, составляя в среднем 1,00 мкл. Это снижение дыхания на 53,5% статистически достоверно ($p<0,001$).

Таблица 3

Интенсивность дыхания мышцы сердца кроликов под влиянием длительного введения глицерина

	Поглощение кислорода в мкл на 1 мг сухого веса ткани		
	контроль		опыт
1	1,86		1,23
2	1,80		0,76
3	3,06		0,60
4	2,00		0,95
5	2,37		1,12
6	1,61		0,97
7	2,26		0,69
8	2,33		0,94
9	2,18		1,41
10			1,38
M	2,16		1,005
±m	0,14		0,088
P		0,0007	

Таблица 4

Интенсивность тканевого дыхания мозга под влиянием длительного введения кроликам глицерофосфата кальция

	Поглощение кислорода в мкл на 1 мг сухого веса ткани		
	контроль		опыт
1	2,93		2,78
2	3,18		2,62
3	4,32		2,16
4	3,15		3,18
5	3,25		3,41
6	2,70		2,90
7	3,28		2,03
8	3,35		2,47
9	3,08		—
M	3,25		2,69
±m	0,15		0,17
P		0,01	

Опыты по изучению влияния глицерина, таким образом, показывают, что будучи введен в небольших дозах в течение длительного времени, он существенно тормозит процесс тканевого дыхания в головном мозгу, печени и особенно заметно — в мышце сердца.

Изучение действия вводимого животным глицерофосфата кальция мы также начали с ткани головного мозга. В опыт брали, как и прежде, левое полушарие. Данные опытов приведены в таблице 4.

Результаты опытов с введением глицерофосфата кальция

приведенные в таблице 4, показывают, что и в этом случае дыхание ткани мозга изменилось по сравнению с контролем. Если поглощение кислорода в контроле было равно в среднем 3,25 мкл, то после длительного введения глицерофосфата кальция оно составило в среднем 2,69 мкл, снизившись на 17,2%. Эта разница статистически существенно выражена ($p=0,01$).

Следующим органом, который подвергался изучению, была печень. В таблице 5 приводятся результаты определений активности тканевого дыхания печени в норме и после длительного введения кроликам глицерофосфата кальция.

Таблица 5

Интенсивность тканевого дыхания печени под влиянием длительного введения кроликам глицерофосфата кальция

	Поглощение кислорода в мкл на 1 мг сухого веса ткани		
	контроль		опыт
1	1,71		1,13
2	1,30		1,35
3	2,31		1,27
4	1,97		1,59
5	1,94		1,98
6	1,33		1,27
7	1,42		1,39
8	2,31		1,82
9	2,43		—
M	1,80		1,47
±m	0,15		0,104
P		0,05	

Таблица 6

Интенсивность тканевого дыхания мышцы сердца под влиянием длительного введения кроликам глицерофосфата кальция

	Поглощение кислорода в мкл на 1 мг сухого веса ткани		
	контроль		опыт
1	1,86		1,47
2	1,80		1,05
3	3,06		1,52
4	2,00		1,60
5	2,37		1,92
6	1,61		1,28
7	2,26		1,39
8	2,33		1,34
9	2,18		
M	2,16		1,45
±m	0,14		0,08
P		0,001	

Как видно из данных таблицы 5, поглощение кислорода тканью печени составило в опыте в среднем 1,47 мкл, то есть снизилось по сравнению с контролем на 21%. При статистической обработке это снижение оказалось существенным: $p=0,05$.

Наконец, последним органом, который мы подвергали исследованию, было сердце. В опыт брался кусочек мышцы левого желудочка сердца. Величины поглощения кислорода мышцей сердца после длительного введения кроликам глицерофосфата кальция приведены в таблице 6.

На основании результатов исследований, помещенных в таблице 6, нетрудно заметить, что в мышце сердца под влиянием введенного глицерофосфата кальция произошло значительное изменение интенсивности тканевого дыхания. Поглощение кис-

лорода с 2,16 мкл в норме снизилось до 1,45 мкл у подопытных кроликов. Эта величина снижения (32,9%) при статистическом анализе оказалась вполне достоверной ($p=0,001$).

Обобщая данные, которые мы получили в опытах с длительным введением кроликам глицерофосфата кальция, можно заключить, что глицерофосфат кальция также снижает интенсивность важнейшего процесса в организме — тканевого дыхания. Это снижение, однако, выражено слабее, чем при действии свободного глицерина, что связано, по-видимому, с присутствием в молекуле глицерофосфата фосфорной кислоты, благодаря которой он активнее включается в обмен веществ и быстрее окисляется, чем свободный глицерин.

Выводы

1. Введение здоровым кроликам глицерина ежедневно в течение 5 месяцев в дозе 50 мг на 1 кг веса животного приводит к угнетению тканевого дыхания головного мозга — на 16,7% ($p=0,001$), печени — на 39,8% ($p=0,001$) и мышцы сердца — на 53,5% ($p<0,001$).

2. Введение здоровым кроликам в той же дозе и при той же продолжительности глицерофосфата кальция как продукта, из которого превращается в организме глицерин, также приводит к угнетению тканевого дыхания головного мозга — на 17,2% ($p=0,01$), печени — на 21,0% ($p=0,05$) и мышцы сердца — на 32,9% ($p=0,001$).

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RATE OF TISSUE RESPIRATION OF CERTAIN ORGANS UNDER THE INFLUENCE OF LONG-TERM ADMINISTRATION OF CHOLESTEROL AND GLYCEROPHOSPHATE TO RABBITS

L. V. Malysheva

It was previously established by us (1,2) that long-term feeding of cholesterol, glycerine, or calcium glycerophosphate given separately to healthy rabbits leads to a decrease in tissue respiration. In addition, it was found that introducing calcium glycerophosphate in feed over a period of five months caused not only a decrease in tissue respiration but other changes as well in the exchange of matter, in particular in lipid exchange and morphologic changes in aorta tissue, corresponding to atherosclerotic changes (3).

In this connection, it is interesting to study the tissue respiration rate of certain organs in rabbits under the influence of long-term introduction of calcium glycerophosphate in feed, during the simultaneous feeding of cholesterol to the animals.

METHODOLOGY

Adult rabbits, of approximately the same age and weight, received daily doses of 0.5 g of cholesterol over a period of four months and 50 mg/kg animal body weight of calcium glycerophosphate. At the end of the period the animals were killed with an air embolism and were subjected to pathologic anatomical examination. In all the rabbits studied, a clearly expressed atherosclerosis of the aorta was found and atherosclerotic changes in other organs were established macro- and microscopically (4).

Rabbits were used as controls which were approximately of the same age and weight, under the same conditions, except they didn't receive either calcium glycerophosphate or cholesterol.

Tissue respiration rates were determined using a Warburg manometer at a temperature of 37.5 deg in an oxygen atmosphere. A calculation was made of oxygen absorption in microliters referred to 1 mg dried tissue weight.

RESULTS OF THE STUDIES

The study of tissue respiration began with the brain. 100 mg of paste made from the left hemisphere were selected for the experiment. The results of the study are presented in Table 1.

As is evident from the data presented in Table 1, the mean value of oxygen absorption in brain tissue of the experimental animals was 2.90 μl, while in the control animals it was 3.25 μl. Consequently, long-term introduction of calcium glycerophosphate along with cholesterol administration somewhat lowered brain tissue respiration activity (10.8 %), which was statistically significant.

TABLE 1. TISSUE RESPIRATION RATE IN RABBIT BRAINS AFTER LONG-TERM ADMINISTRATION OF CHOLESTEROL AND GLYCEROPHOSPHATE

Number	Oxygen absorption in μl/1 mg dried tissue: Control	Experimental
	2,93	2,56
	3,18	3,24
	4,32	3,38
	3,15	2,78
	3,25	2,23
	2,70	3,02
	3,28	2,91
	3,35	3,08
	3,08	
	3,25	2,90
	0,15	0,13
		0,05

TABLE 2. TISSUE RESPIRATION RATE IN RABBIT LIVERS AFTER LONG-TERM ADMINISTRATION OF CHOLESTEROL AND GLYCEROPHOSPHATE

Number	Oxygen absorption in μl/1 mg dried tissue: Control	Experimental
1	1,74	1,41
2	1,30	1,57
3	2,31	1,36
4	1,97	0,74
5	1,94	0,93
6	1,33	1,44
7	1,42	1,57
8	2,31	2,09
9	2,43	
M	1,86	1,39
m	0,15	0,14
P		0,01

To determine the tissue respiration rate of liver, 220 mg portions of a paste made of the left interior section was placed in a Warburg vessel.

The results of the studies presented in Table 2 show that glycerophosphate administration with cholesterol in the majority of experiments leads to a significant decrease in oxygen absorption in liver tissue. If oxygen absorption in the control group equals 1.86 μl, in the experimental group it was only 1.39 μl. The indicated difference was statistically reliable ($p = 0.01$).

Finally, the rate of tissue respiration in rabbit heart muscle was studied. 100 mg batches of paste were selected for the experiment. The results of these studies are set forth in Table 3.

TABLE 3. TISSUE RESPIRATION RATE OF RABBIT HEART MUSCLE DURING LONG-TERM CHOLESTEROL AND GLYCEROPHOSPHATE ADMINISTRATION

Number	Oxygen absorption in μl/1 mg dried tissue: Control	Experimental
1	1,86	1,66
2	1,80	1,10
3	3,16	1,43
4	2,00	1,11
5	2,37	1,22
6	1,61	0,95
7	2,26	0,90
8	2,33	1,44
9	2,18	
M	2,16	1,22
m	0,14	0,09
P		0,001

Oxygen absorption in heart muscle tissue under the influence of cholesterol and glycerophosphate is decreased from 2.16 μl in the control group to 1.22 μl in the experimental animals. The decrease in heart muscle respiration was 43.5 %. During the statistical analysis, these data proved to be completely reliable ($p < 0.001$).

Thus, in the study of tissue respiration rate in certain organs in rabbits after long-term administration of cholesterol and glycerophosphate with feed, which leads to experimental atherosclerosis, a significant decrease was found in it: 10.8 % in the brain, 25.3 % in the liver, and 43.5 % in heart muscle. Considering these data, it is evident that respiration rate in heart muscle and liver decreased to a large extent, compared to rabbits which received only calcium glycerophosphate in their feed.

(Both series of experiments were conducted on rabbits of approximately the same age and weight, which removed the effect of these factors on the process of tissue respiration.) Therefore the results obtained can be explained as the total action of cholesterol and calcium glycerophosphate introduced over a long time on exchange of matter, and in particular on tissue respiration.

In brain tissue however a less clearly expressed decrease in tissue respiration was observed than in other organs in this series of experiments. This is evidently associated with the fact that in the formation period of experimental atherosclerosis, they are reflected mainly by vascular tissue (aorta), heart muscle, liver, and other organs.

CONCLUSIONS

1. During atherosclerosis evoked by long-term administration of cholesterol in association with calcium glycerophosphate, a significant decrease in respiration rate was observed: 10.8 % in the brain ($p = 0.05$), 25.3 % in the liver ($p = 0.01$), 43.5 % in heart muscle ($p = 0.001$).

2. The decrease in tissue respiration in the organs of experimental animals studied is a result of the total action of long-term administration to rabbits of cholesterol and calcium glycerophosphate.

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ИНТЕНСИВНОСТЬ ТКАНЕВОГО ДЫХАНИЯ НЕКОТОРЫХ ОРГАНОВ ПОД ВЛИЯНИЕМ ДЛИТЕЛЬНОГО ВВЕДЕНИЯ КРОЛИКАМ ХОЛЕСТЕРИНА И ГЛИЦЕРОФОСФАТА

Л. В. МАЛЫШЕВА

Ранее нами установлено (1, 2), что длительное кормление здоровых кроликов холестерином, глицерином или глицерофосфатом кальция в отдельности ведет к выраженному угнетению тканевого дыхания. Кроме того, найдено, что введение с пищей глицерофосфата кальция в течение 5 месяцев вызывает не только угнетение тканевого дыхания, но и другие изменения в обмене веществ, в частности в липоидном обмене, а также и морфологические изменения в ткани аорты, соответствующие атеросклеротическим (3).

В связи с этим, представлялось интересным изучить интенсивность тканевого дыхания некоторых органов кроликов под влиянием длительно вводимого с пищей глицерофосфата кальция при одновременном кормлении животных холестерином.

Методика

Взрослые кролики, примерно одинакового возраста и веса, ежедневно в течение 4 месяцев получали с пищей холестерин в дозе 0,5 г и глицерофосфат кальция в дозе 50 мг/кг веса тела животного. В конце срока животных забивали воздушной эмболией и подвергали патологоанатомическому вскрытию. У всех подопытных кроликов был обнаружен ярко выраженный атеросклероз аорты и атеросклеротические изменения в других органах, установленные макро- и микроскопически (4).

В качестве контроля использовали кроликов, примерно того же возраста и веса, находившихся в тех же условиях, но не получавших ни глицерофосфата кальция, ни холестерина.

Интенсивность тканевого дыхания определяли манометрическим методом в аппарате Варбурга, при температуре 37,5°, в атмосфере кислорода. Расчет производили в микролитрах поглощенного за 1 час кислорода, отнесенных к 1 мг сухого веса ткани.

Результаты исследований

Изучение тканевого дыхания начали с головного мозга. Для опыта брали 100 мг кашицы левого полушария. Результаты исследований представлены в таблице 1.

Как видно из приведенных в таблице 1 данных, средняя величина поглощения кислорода тканью мозга у подопытных животных составляет 2,90 мкл, в то время как у контрольных она равна 3,25. Следовательно, длительное введение глицерофосфата кальция в сочетании с введением холестерина несколько снизило активность дыхания мозговой ткани (10,8%), что статистически является существенным.

Таблица 1

Интенсивность тканевого дыхания мозга кроликов после длительного введения холестерина и глицерофосфата

Поглощение кислорода в мкл на 1 мг сухой ткани		
№№	контроль	опыт
1	2,93	2,56
2	3,18	3,24
3	4,32	3,38
4	3,15	2,78
5	3,25	2,23
6	2,70	3,02
7	3,28	2,91
8	3,35	3,08
9	3,08	
M	3,25	2,90
m	0,15	0,13
P		0,05

Таблица 2

Интенсивность тканевого дыхания печени кроликов после длительного введения холестерина и глицерофосфата

Поглощение кислорода в мкл на 1 мг сухого веса ткани		
№№	контроль	опыт
1	1,74	1,41
2	1,30	1,57
3	2,31	1,36
4	1,97	0,74
5	1,94	0,93
6	1,33	1,44
7	1,42	1,57
8	2,31	2,09
9	2,43	
M	1,86	1,39
m	0,15	0,14
P		0,01

Для определения интенсивности дыхания ткани печени в сосудик Варбурга погружали 200 мг кашицы левой внутренней ее доли.

Результаты исследований, приведенные в таблице 2, показывают, что введение глицерофосфата вместе с холестерином в

Большинство опытов приводит к значительному снижению поглощения кислорода печеночной тканью. Если поглощение кислорода в контроле равно 1,86 мкл, то в опыте оно составляет лишь 1,39 мкл. Указанная разница статистически достоверна ($p = 0{,}01$).

Наконец, была изучена интенсивность тканевого дыхания мышцы сердца кроликов. Для опыта брали навеску кашицы весом в 100 мг. Результаты этих исследований приводятся в таблице 3.

Таблица 3

Интенсивность тканевого дыхания мышцы сердца кроликов при длительном введении холестерина и глицерофосфата

№	Поглощение кислорода в мкл на 1 мг сухого веса ткани	
	контроль	опыт
1	1,86	1,66
2	1,80	1,10
3	3,66	1,43
4	2,00	1,11
5	2,37	1,22
6	1,61	0,95
7	2,26	0,90
8	2,33	1,34
9	2,18	
M	2,16	1,22
m	0,14	0,09
P		0,001

Поглощение кислорода тканью мышцы сердца под влиянием холестерина и глицерофосфата снижается с 2,16 мкл в контроле до 1,22 мкл у подопытных кроликов. Угнетение дыхания сердечной мышцы составляет 43,5%. При статистической обработке эти данные оказались вполне достоверными ($p < 0{,}001$).

Таким образом, при исследовании интенсивности тканевого дыхания некоторых органов кроликов после длительного введения с пищей холестерина и глицерофосфата, приводящего к экспериментальному атеросклерозу, найдено значительное его угнетение: в головном мозге — на 10,8%, в печени — на 25,3% и в мышце сердца — на 43,5%. При рассмотрении этих данных видно, что в мышце сердца и печени интенсивность дыхания снижена в большей степени, чем у кроликов, получавших с пищей один глицерофосфат кальция. (Обе серии опытов проводились на кроликах примерно одинакового возраста и веса, что устраняло влияние этих факторов на процесс тканевого дыхания). Поэтому полученные результаты можно объяснить суммарным действием вводимых длительно холестерина и глицерофосфата кальция на обмен веществ, и в частности на тканевое дыхание.

В ткани головного мозга, однако, наблюдается менее выраженное угнетение тканевого дыхания, чем в других органах этой серии животных. Это, по-видимому, связано с тем, что в период сформировавшегося экспериментального атеросклероза поражаются, главным образом, сосудистая ткань (аорта), мышца сердца, печень и другие органы.

Выводы

1. При атеросклерозе, вызванном длительным введением холестерина в сочетании с глицерофосфатом кальция, наблюдается значительное угнетение интенсивности тканевого дыхания: в головном мозгу — на 10,8% ($p=0,05$), печени — на 25,3% ($p=0,01$), мышце сердца — на 43,5% ($p=0,001$).

2. Угнетение тканевого дыхания в исследуемых органах опытных кроликов является результатом суммарного действия длительно вводимого кроликам холестерина и глицерофосфата кальция.

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Zeitschrift für Krebsforschung 52, 32-6 (1942)

ON THE EFFECT OF CHEMICAL STIMULATION ON THE GROWTH OF MOUSE TUMORS

(Part II)

by

M. Popoff

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Director: Prof. Dr. M. Popoff)

With 1 figure

(Received on May 16, 1941)

In my publication "On the Effect of Some Chemical Stimulants on the Growth of Cancer Tumors" (Acta Cancrologica 2, No. 2, 173-180 (1936)), I discussed the theoretical considerations which suggest that it must be possible, by elevating the general cell functions of the body and its endocrine glands with stimulants, to normalize the chemical conversions and the growth correlations of the body, and in this way to hem the growth and increase of the tumor cells. As support of this concept, I related the results of experiments with $MgCl_2$, $MgSO_4$, MgBr, KBr, glycerine, $MnSO_4$, KI and $NaSO_4$ involving both exterior use on cancer lesions and subcutaneous injections. They revealed a clear inhibition of the tumor growth, but without harming the vitality of the tumor cells to such an extent that the growth of the tumor was completely suspended. In the light of this fact, some functional alterations which favor the life of the tumor cells and make it possible for them to survive must have been left in the body. The growth regulation processes of the body could not be reversed completely to their normal direction because of these influences.

Departing from these findings, I attempted further to intensify the observed inhibitions of growth of tumor cells by varying different stimulants both according to concentration and according to composition. As the results to be reported here will show, I succeeded in achieving a significantly more intensive inhibition of growth, which suggests that there are and must be chemical influences that are capable of affecting tumor growth to a great extent, and that as a consequence it would be worthwhile to research this matter further. However, I did not succeed this time either in suspending tumor growth completely.

The methodology was as follows:

After many experiments with differently varied stimulant solutions, the following composition gave me, relatively speaking, the best inhibiting effects. The keynotes of stimulant composition are presented in detail in my book, Cell Stimulation and its Application in Plant Cultivation and Medicine (Paul Parey Publishers, Berlin, 1931, p. 375).

0.5 g $MgCl_2$ + 200 mg $MnSO_4$. -- These two chemical substances were established by me as generally cell stimulatory, according to earlier detailed examinations. The same is true of glycerine -- 2 ccm, calcium glycerophosphoricum -- 15 drops and iodine -- 3 drops of normal tincture of iodine. LiCl and LiJ were also included in the solution as general cell stimulants, and were mixed in in the amount of 3 ccm each in a 5% solution. The lithium salts especially were also included for the reason that, according to the research of Herbst with sea urchin eggs and larvae, lithium exercises a regulatory influence on growth and therefore to a certain degree promotes the organic formative stimuli of the body. In order to increase the activity of the sex glands as important, endocrine glands, a small dose -- 2 ccm of a 1% iohimbine solution -- was added to the above stimulants. This was all dissolved in clear, filtered 40 ccm boiled earth. It has been shown in cultivation experiments with protozoa that humus acids exercise a favorable influence on the normalization of cell functions. Now, in order to add vitamins to this solution, I added 5 ccm of an extract of sharp, red pepper husks. This extract was produced in the following way: 2 g of air-dried pepper husks were ground finely and extracted in 50 ccm water at a temperature of 60-70° for 20 minutes in an enameled flask. After depositing, 5 ccm of this extract were added to the solution. Often repeated experiments with injections of pepper extract alone have also yielded a certain inbiting effect on the growth of tumors. It is possible that in this case capsaicin played a certain role. Research in this direction is not yet complete.

The solution thus prepared was produced fresh before each use.

The experiments were performed as follows: Each series of experiments involved 15-20 white mice, transplanted subcutaneously with pieces of the same tumor. After 3-4 days, when the tumor had begun to grow, the tumor mice were divided into two equal groups according to size of the tumors. One of these groups was then used as a control group and was not treated. The other group was injected subcutaneously with the stimulant solution (½ ccm), and always on that side of the body opposite to the tumor. The injections were given 2-3 consecutive days, once a day, then suspended for a day, started again, etc. A total of 8-10 injections were given. At the end of this time, considerable differences in tumor growth could already be seen between the controls and the experimental animals. These differences were often very striking, as shown in the photograph (figure 1). These experiments were performed on about 300 mice, and inhibition of growth could always be observed. The tumors of the treated mice were from 1/3 to 1/5 the size of the tumors of the control mice.

Inhibition of growth continued as long as the injections were continued. After suspension of the injections, growth began anew,

which speaks for the fact that the tumor cells were inhibited in their division by the injected solution, but not so extensively damaged that they had completely suspended their growth. As a consequence, the treated mice survived the controls by at most 1-3 weeks and then also perished from the tumors growing again. The mice did not tolerate too often repeated injections, for when used for a long time, the solution gradually revealed harmful effects on the animal.

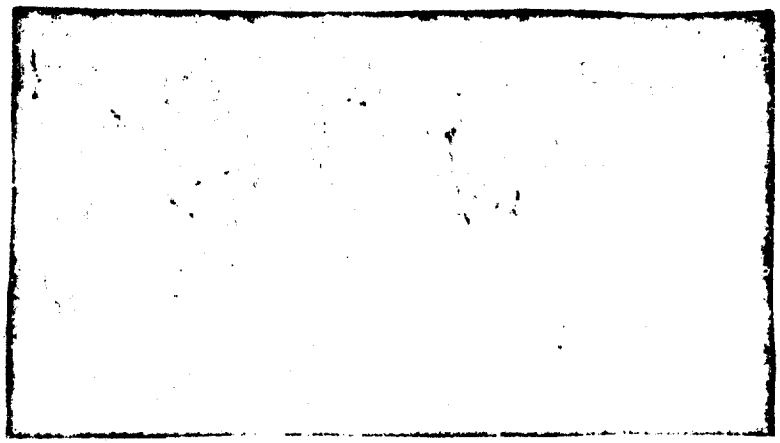

Figure 1. Upper row: untreated mice tumors -- controls. Bottom row: treated mice tumors from the same experimental series.

These experiments show that it is possible to inhibit tumor growth extensively for a certain period of time -- in fact, for as long as the injections continue. These results, even if they are at present still incomplete, support the hope that, by improving the injection solution, it could be possible to increase further the growth-inhibiting effect and to make it more lasting. At the same time, we must strive to replace the metal salts with organic Mg and Li compounds. According to my previous experience, it seems that the magnesium, manganese, and especially the lithium salts have a clearly inhibiting effect on tumor growth, and not only through injections, but also through oral intake, through direct feeding.

Experiments in this direction involved about 200 mice and were carried out in experimental series of 14-20 mice each, the tumors of which stemmed in this case as well from one original tumor. In this case, too, half the mice were left untreated for controls, and the other half was used as experimental animals. Feeding of both groups was measured strictly as concerned the amount of food: both the controls and the experimental animals received the same amount of boiled milk, bread and oats. The only difference was that the experimental mice received a supplement of lithium and manganese salts in their milk, 1 ccm of a 10% LiCl solution and 0.3 ccm of a 10% $MnSO_4$ solution per 100 ccm milk. Every third day, beyond this, 1 drop of tincture of iodine was added to the Li-Mn-milk. The mice that received this food demonstrated a clear inhibition of tumor growth; the tumor reached

1/3 to 1/4 normal size in comparison with the control mice. The inhibiting effect was visible. Since the mice did not like to eat the Li-Mn-milk for a long time, we included a recovery day with normal milk after every 3-4 feeding days. However, in this case as well, the sum effect of the Li-Mn-effect was a damaging one, so that I had a mortality rate of 30-40% among the experimental animals. This high mortality I attempted to reduce by lowering the Li-Mn concentration; this was successful, but at the same time, the favorable, inhibiting effect of the Li-Mn salts on tumor growth decreased considerably.

The mice subjected to the treatment demonstrated a diminution in tumor growth but survived the control mice by only 1-3 weeks -- they died gradually one after the other.

Also noteworthy in these experiments was the fact that the tumors manifested only an inhibition of growth: the living capacity of the tumor cells was preserved, since after cessation of treatment the growth of the tumor began anew and, as in the case of the control mice, though about 2-3 weeks later, reached a stage of ulceration and led to the death of the mice.

These experiments also showed that it is necessary to achieve a decrease in the harmful effect of the Li-Mn-salt solutions, in order to be able to better utilize the growth-inhibiting effect of the Li-Mn compounds.

It is possible that organic compounds will become significant in this area.

Summary

In this second report (the first part is in Acta Cancrologica 2, No. 2 (1936), the experiments demonstrating the effect of chemical stimulants on the growth of mouse tumors are described. Solutions of $MgCl_2$, $MnSO_4$, Ca glycerophosphate, iohimbine and iodine and capsaicin in the form of subcutaneous injections are used as stimulants. In the case of the percentual composition of these solutions indicated in the text, a clearly inhibiting effect on tumor growth could be observed.

An inhibiting effect on tumor growth could also be achieved by feeding LiCl and $MnSO_4$. However, in both cases, the tumor later began to grow anew -- after suspension of the treatment. Thus, the tumor cells are only impeded in their increase, but are not lastingly damaged.

The administration of all these chemical substances in the optimally effective stimulation dose was intended to normalize the life functions of the body cells and the activity of the endocrine glands, to increase both of these, and in this way to direct the body tissue to ordered growth correlations. Experiments in this direction are being continued.

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(Aus dem Biologischen Institut der Universität Sofia [Bulgarien].
Direktor: Prof. Dr. *Methodi Popoff*.)

Über die Wirkung chemischer Stimulationsmittel auf das Wachstum von Mäusetumoren.

(II. Mitteilung.)

Von

M. Popoff.

Mit 1 Textabbildung.

(*Eingegangen am [illegible] Mai 1941.*)

In meiner Publikation „Über die Wirkung einiger chemischer Stimulationsmittel auf das Wachstum von Krebstumoren" [Acta Cancrologica 2, H. 2. 173—180 (1936)], habe ich die theoretischen Erwägungen besprochen, welche dahin weisen, daß durch eine Hebung der allgemeinen Zellfunktionen des Körpers und seiner innersekretorischen Drüsen durch Stimulationsmittel möglich sein muß, die chemischen Umsetzungen und die Wachstumskorrelationen des Körpers wieder zu normalisieren und auf diese Weise das Wachstum und die Vermehrung der Tumorzellen einzudämmen. Zur Stützung dieser Auffassungsweise habe ich die Resultate von Versuchen mit $MgCl_2$, $MgSO_4$, MgBr, KBr, Glycerin, $MnSO_4$, KJ und $NaSO_4$ sowohl bei äußerer Anwendung auf Krebswunden als auch bei subcutanen Einspritzungen angeführt. Sie zeigten eine deutliche Hemmung des Tumorwachstums, ohne aber die Vitalität der Tumorzellen so weit zu schädigen, daß der Tumor sein Wachstum vollständig einstellte. Demnach müßten im Körper noch Funktionsumstimmungen geblieben sein, die das Leben der Tumorzellen begünstigten und möglich machten. Die Wachstumsregulationsprozesse des Körpers haben durch diese Einwirkungen nicht ganz nach der normalen Richtung umgestimmt werden können.

Von diesen Konstatierungen ausgehend habe ich versucht, durch Variieren verschiedener Stimulationsmittel sowohl nach Konzentration als auch nach Zusammensetzung die beobachteten Wachstumshemmungen auf die Tumorzellen weiter zu verstärken. Wie die hier mitzuteilenden Ergebnisse zeigen werden, gelang es mir, in der Tat eine bedeutend stärkere Hemmung des Wachstums zu erreichen, was darauf hindeutet, daß es chemische Einwirkungen gibt und geben muß, die imstande sind, das Tumorwachstum weitgehend zu beeinflussen und daß es folglich lohnend wäre, in dieser Richtung weiterzuforschen. Ein vollständiges Aufheben des Tumorwachstums gelang mir auch diesmal nicht.

Nachfolgend die Arbeitsweise:

Nach vielen Versuchen mit verschiedentlich variierten Stimulationslösungen hat mir die folgende Zusammenstellung verhältnismäßig die besten Hemmungswirkungen gegeben. Die Leitgedanken der Stimulationszusammenstellungen sind in meinem Buch „Die Zellstimulation und ihre Anwendung in der Pflanzenzüchtung und Medizin" (Verlag Paul Parey, Berlin, 1931, S. 375) ausführlich dargelegt.

0,5 g $MgCl_2$ + 200 mg $MnSO_4$. — Diese zwei chemischen Mittel sind nach früheren eingehenden Untersuchungen von mir als allgemein zellstimulationswirkend erkannt worden, desgleichen auch das Glycerin — 2 ccm, das Calcium Glycerophosphoricum — 15 Tropfen und das Jod — 3 Tropfen normale Jodtinktur. Ebenfalls als allgemeine Zellstimulationsmittel wurden das LiCl und das LiJ in der Lösung aufgenommen, und zwar zu je 3 ccm von einer 5proz. Lösung. Speziell die Lithiumsalze wurden außerdem noch aus dem Grunde aufgenommen, da nach den Untersuchungen von *Herbst* an Seeigeleiern und Seeigellarven das Lithium eine regulatorische Wirkung auf die Wachstumsprozesse ausübt und so gewissermaßen die organischen formativen Reize des Körpers fördert. Um die Tätigkeit der Geschlechtsdrüsen als wichtige, inkretorische Drüsen zu heben, wurde den obigen Stimulationsmitteln auch eine geringe Dosis — 2 ccm einer 1proz. Johimbinlösung zugesetzt. Das Ganze wurde in klar filtrierte 40 ccm Erdabkochung aufgelöst. Aus Züchtungsversuchen an Protozoen ist bekannt geworden, daß die Humussäuren eine günstige Wirkung auf die Normalisierung der Zellfunktionen ausüben. Um nun die so zusammengesetzte Lösung vitaminhaltig zu gestalten, habe ich 5 ccm von einem Extrakt von scharfen, roten Pfefferschoten zugesetzt. Dieser Extrakt wurde auf folgende Weise hergestellt: 2 g luftgetrocknete Pfefferschoten wurden fein verrieben und in 50 cmm Wasser bei Temperatur von 60—70° 20 min lang in emailliertem Gefäß extrahiert. Nach dem Absetzen wurden von diesem Extrakt 5 ccm der Lösung zugegeben. Vielfach wiederholte Versuche mit Injektionen von Pfefferextrakt allein haben auch eine gewisse Hemmung auf das Tumorenwachstum ergeben. Möglicherweise hat hier das Capsaicin eine gewisse Rolle gespielt. Die Untersuchungen in dieser Richtung sind noch nicht abgeschlossen.

Die so zusammengesetzte Lösung wurde jedesmal vor der Anwendung frisch hergestellt.

Die Versuche wurden folgendermaßen ausgeführt: Jede Versuchsserie bestand aus 15—20 weißen Mäusen, die subcutan mit Stückchen von demselben Tumor transplantiert worden waren. Nach 3—4 Tagen, wenn der Tumor zu wachsen begonnen hatte, wurden die Tumormäuse in zwei, entsprechend der Tumorgröße gleiche Teile geteilt. Der eine davon wurde ohne Behandlung als Kontrolle weitergeführt. Der andere

Teil wurde mit Stimulationslösung ($^1/_2$ ccm) subcutan injiziert, und zwar jedesmal auf der dem Tumor entgegengesetzten Körperhälfte. Die Injektionen wurden 2—3 Tage nacheinander einmal täglich unternommen, dann einen Tag ausgesetzt, wieder injiziert usw. Es wurden im ganzen 8—10 Injektionen gegeben. Nach Ablauf dieser Zeit konnten schon erhebliche Unterschiede im Tumorwachstum zwischen den Kontrollen und den Versuchstieren festgestellt werden, die vielfach sehr auffallend waren, wie die Photographie (Abb. 1) zeigt. Diese Versuche wurden an etwa 300 Mäusen unternommen, die Wachstumshemmung

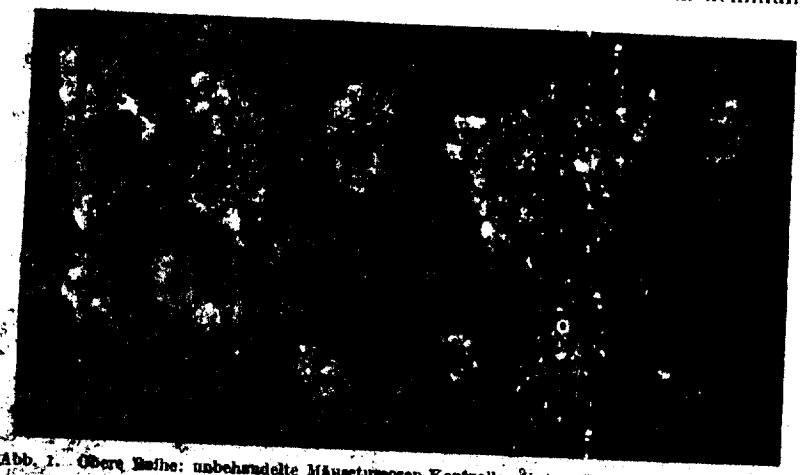

Abb. 1. Obere Reihe: unbehandelte Mäusetumoren-Kontrolle. Untere Reihe: behandelte Mäusetumoren von der gleichen Versuchsserie.

war stets zu beobachten. Die Tumore der behandelten Mäuse bildeten nachdem $^1/_3$ bis $^1/_5$ der Tumorgröße der Kontrollmäuse.

Die Wachstumshemmung dauerte so lange an, als die Injektionen fortgesetzt wurden. Nach Einstellung derselben fing das Wachsen von neuem an, was dafür spricht, daß die Tumorzellen in ihrer Teilung durch die injizierte Lösung gehemmt waren, nicht aber so weitgehend geschädigt, daß sie ihr Wachstum vollständig eingestellt hätten. Infolgedessen überlebten die behandelten Mäuse die Kontrollen mit höchstens 2—3 Wochen und gingen dann an den nachwachsenden Tumoren ebenfalls zugrunde. Zu oft wiederholte Injektionen vertrugen die Mäuse nicht, denn längere Zeit angewandt, zeigte die Lösung allmählich schädigende Wirkungen für das Tier.

Diese Versuche zeigen, daß es gelingt, für eine gewisse Zeit das Tumorwachstum weitgehend zu hemmen — und zwar so lange, wie die Injektionen dauern. Diese, wenn auch zur Zeit noch unvollkommenen Resultate geben aber der Hoffnung Raum, daß es nach dieser Richtung,

durch Verbesserung der Injektionslösungen möglich sein könnte, die wachstumshemmende Wirkung noch weiter zu steigern und dauerhafter zu machen. Es muß dabei angestrebt werden, die Metallsalze durch organische Mg- und Li-Verbindungen zu ersetzen. Nach meinen bisherigen Erfahrungen scheint es mir, daß die Magnesium-, Mangan- und besonders die Lithiumsalze eine deutlich hemmende Wirkung auf das Tumorwachstum haben, und zwar nicht nur auf dem Wege der Injektionen, sondern auch durch Einnahme per os, durch direkte Verfütterung.

Die Versuche nach dieser Richtung belaufen sich auf etwa 200 Mäuse, durchgeführt in Versuchsserien von je 14—20 Mäusen, deren Tumoren auch hier jedesmal von einem Ausgangstumor abstammten. Auch hier wurde die eine Hälfte der Mäuse als Kontrolle unbehandelt gelassen und die andere Hälfte als Versuchstiere verwendet. Die Fütterung beider Gruppen war streng abgemessen nach der Menge der Nahrungsmittel: sowohl die Kontrollen als auch die Versuchstiere bekamen dasselbe Quantum abgekochter Milch, Brot und Hafer. Der einzige Unterschied bestand darin, daß die Versuchsmäuse in die Milch einen Zusatz von Lithium- und Mangansalzen erhielten, und zwar auf 100 ccm Milch je 1 ccm von einer 10proz. LiCl-Lösung und 0,3 ccm von einer 10 proz. $MnSO_4$-Lösung. An jedem dritten Tag wurde außerdem der Li-Mn-Milch auch je 1 Tropfen Jodtinktur zugesetzt. Die Mäuse, die diese Nahrung bekamen, zeigten eine deutliche Hemmung des Tumorwachstums, dieses erreichte im Vergleich zu den Kontrollmäusen $^1/_3$ bis $^1/_4$ der normalen Größe. Die Hemmungswirkung war augenscheinlich. Da die Mäuse die Li-Mn-Milch auf die Dauer nicht gerne nahmen, so wurde je nachdem nach 3—4 Fütterungstagen ein Erholungstag mit normaler Milch eingeschaltet. Durch die Summierung der Li-Mn-Wirkung kam es aber auch hier wie bei den Injektionsversuchen schließlich zu einer schädigenden Wirkung, so daß ich eine Sterblichkeit der Versuchstiere von 30—40% hatte. Diese hohe Sterblichkeit habe ich versucht durch Herabsetzung der Li-Mn-Konzentration zu vermindern, was mir auch gelang, gleichzeitig damit aber nahm auch die günstige, hemmende Wirkung der Li-Mn-Salze auf das Tumorwachstum beträchtlich ab.

Die Mäuse, welche die Behandlung überstanden und eine Herabsetzung des Tumorwachstums zeigten, überlebten die Kontrollmäuse mit nur 1—3 Wochen — sie starben allmählich eine nach der anderen ab.

Bemerkenswert auch bei diesen Versuchen war, daß die Tumoren nur eine Wachstumshemmung zeigten: die Lebensfähigkeit der Tumorzellen blieb erhalten, da nach dem Aufhören der Behandlung das Wachstum des Tumors von neuem anfing, um schließlich wie bei den Kontrollmäusen, wenn auch mit einer Verspätung von 2—3 Wochen, das Stadium der Ulceration zu erreichen und den Tod der Mäuse herbeizuführen.

logica 2, H. 2 (1950)

wirkung von chemischen Stimulation...

Mäusetumoren zeigen. Als Stimulationsmittel werden ...

von $MgCl_2$, $MnSO_4$, Ca-Glycerophosphat, Johimbin, J und Capsalcin angewandt, und zwar als subcutane Injektionen. Bei der im Text angegebenen prozentualen Zusammensetzung dieser Lösungen konnte eine deutlich hemmende Wirkung auf das Tumorwachstum beobachtet werden.

Eine hemmende Wirkung des Tumorwachstums wurde auch erzielt bei Verfütterung von LiCl und $MnSO_4$. In beiden Fällen fing aber das Wachstum des Tumors später von neuem an — nach der Aufhebung der Behandlung. Also werden die Tumorzellen in ihrer Vermehrung nur aufgehalten, aber nicht dauernd geschädigt.

Die Darreichung aller dieser chemischen Mittel in der optimal wirkenden Stimulationsdosis hatte den Zweck, die Lebensfunktionen der Körperzellen und die Tätigkeit der innersekretorischen Drüsen zu normalisieren und zu heben und auf diese Weise das Körpergewebe wieder zu geordneten Wachstumskorrelationen zurückzuführen. Die Versuche in der hier angegebenen Richtung werden weiter fortgeführt.

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Cariostatic Effect of Glycerophosphate

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Abstract.—The effects of calcium or sodium α- or β-glycerophosphate (GP), basic sodium phosphate, fluoride or calcium chloride on caries, plaque and calculus formation and on eating patterns was investigated in a short-term rat caries test. The compounds were added to the cariogenic diet at a 0.6% P level or at a level equimolar to 0.6% P. Smooth surface and fissure lesions were inhibited by all phosphates but enhanced by calcium chloride. The group receiving $CaCl_2$ had the longest eating times. There were significant but small differences in plaque formation. Calculus formation was enhanced by all test compounds. Growth was strongly inhibited by Ca-GP and $CaCl_2$.

Key words: Phosphate—Glycerophosphate—Calcium—Caries in rats—Calculus in rats

Introduction

Osborn, Noriskin and Statz (1937) postulated that crude sugars and cereals contain a "tooth protective agent" which is partially lost during the refining processes. In the search for this "protective agent" Osborn *et al.* (1937) and Osborn (1941) investigated the effects of the addition of various organic and inorganic calcium phosphates to teeth-sucrose-saliva mixtures. Addition of calcium glycerophosphate to this system resulted in drastic inhibition of enamel dissolution. Jenkins, Forster and Speirs (1959) reinvestigated quantitatively Osborn's work (Osborn, 1941; Osborn *et al.*, 1937) using more standardized techniques and homogenous ground materials and confirmed the statement of Osborn *et al.* (1937) that certain organic phosphates lower the solubility of calcium phosphates. The cariostatic effect of glycerophosphate was tested in several animal studies. In rat experiments, addition to the diet of sodium glycerophosphate at a concentration of 0.29% P (König, Marthaler and Mühlemann, 1961) or calcium glycerophosphate at the 0.48% P level (Lilienthal *et al.*, 1966) resulted in small but not significant caries reductions. In other experiments, however, sodium glycerophosphate decreased by more than 70% the caries incidence of rats fed a diet containing this compound at the 0.49% P level (McClure, 1963, 1964). In hamsters the caries reducing effect of sodium glycerophosphate was about the same as that of sodium fluoride (Plathner and Winiker, 1971). Bowen (1969) tested the buffering properties of glycerophosphate in monkeys, and found that inclusion of 0.25 and 1% calcium glycerophosphate in 1/10 M sucrose was effective in buffering acid production in plaque. Monkeys receiving icing sugar containing 1% calcium glycerophosphate 5 times daily developed only a total of 5 lesions in 22 experimental months, while the control animals had a total of 47 carious lesions (Bowen, 1971a, b). Purpose of the present rat experiment was to determine the cariostatic effects of various glycerophosphates added at the 0.6% P level to a cariogenic diet.

Material and methods

Animals and general procedures

The experiment was conducted on 10 litters of Osborne-Mendel rats, each litter consisting of 9 animals.

In order to avoid fissure impaction the animals with their dams were transferred on day 13 from the breeding cages to screen-bottom stainless steel cages without bedding and fed finely (Ø < 0.315 mm) powdered stock diet◊. On day 21 the animals were weaned and transferred to the experimental cages, 2 in each cage. The treatments listed in Table I were distributed to the animals by random numbers. Treatment consisted of administration of diet 2000a◊◊ (56% sucrose, 28% skim-milk, 8% wheat flour, 5% yeast, 2% Gevral Protein® Lederle, 1% sodium chloride) or diet 2000a supplemented with the compounds listed in Table I ata 0.6% P level or at a level equimolar to 0.6% P. Diet and drinking water were available *ad libitum*. The animals were killed after 21 experimental days.

◊ Rat Checkers No. 184. Nafag AG, CH-9202 Gossau SG, Switzerland

◊◊ Total phosphorus content 0.43% P (unsupplemented diet ashed with concentrated sulfuric and nitric acid)

Table 1 List of treatments and solubility of the compounds added to the diet at a 0.6% P level or at a level equimolar to 0.6% P. GP = glycerophosphate

Treatments	Solubility◊ in 100 ml H_2O (g)
1 Control	—
2 Control	—
3 Ca-GP◊◊, 25% α- and 75% β-isomers	2.0
4 Na-GP, 25% α- and 75% β-isomers	v.s.
5 Na-GP, 100% α-isomer◊◊◊	v.s.
6 Na-GP, 100% β-isomer◊◊◊◊	v.s.
7 Na_2HPO_4◊◊◊◊◊	100 (dihydrate)
8 $CaHPO_4 \cdot 2\ H_2O$◊◊◊◊◊	0.03
9 $CaCl_2 \cdot 2\ H_2O$◊◊◊◊◊	97.7

◊ Handbook of Chemistry and Physics, 47th edition, 1966–1967, Ed. R.C. Weast, S.M. Selby. The Chemical Rubber Co., Cleveland, Ohio 44114, USA

◊◊ Grade XII SIGMA, tetrahydrate

◊◊◊ Grade X SIGMA, hexahydrate

◊◊◊◊ Grade I SIGMA, pentahydrate

◊◊◊◊◊ E. Merck AG, D-61 Darmstadt, Germany

Eating pattern evaluation

The eating pattern of the free-fed animals on treatment 1, 3, 4, 7, 8 and 9 was continuously recorded over 5 3-day periods using the device described by KÖNIG *et al.* (1964).

Plaque evaluation

Immediately after sacrifice plaque on the mandibular and maxillary molars was stained by dipping the removed jaws into 1% erythrosin◊◊◊ for 15 sec. Non-plaque material was destained by placing the jaws under running tap water for 5 sec. The extent of buccal and lingual smooth surface plaque on the first and second molars in the 4 quadrants was evaluated under a dissection microscope using a 0–4 grading index. Zero indicated absence of smooth surface plaque, but not necessarily absence of plaque on occlusal surfaces or in fissures. Grade 4 indicated virtually complete covering of the smooth surfaces with plaque. Grade 1, 2, and 3 indicated intermediate stages.

Caries evaluation

After scoring plaque the jaws were fixed in 10% buffered formalin. After fixation, soft tissue was scraped from the mandibles, and the dried buccal surfaces of the first and second lower molars were inspected for smooth surface lesions under a dissection microscope. The extent of smooth surface lesions was recorded on charts and scores attributed using KEYES's (1958) area units E. Afterwards, serial mesiodistal sections were prepared from the mandibles with an internal rim rotary cutting machine (JANSEN, 1950). The sections were stained, serially mounted on slides and randomized prior to evaluation. A total of 12 fissures of the first and second lower molars were examined microscopically for carious lesions according to the Zurich scoring method.

◊◊◊ Erythrosin extra bläulich, Standard Fluka 45690, Fluka AG, CH-9470 Buchs SG, Switzerland

Calculus assessment

The maxillary molars were inspected with the aid of a dissection microscope at 32 × magnification for location and extent of calculus deposits.

Data were recorded blind on charts using a 20-units calculus index (REGOLATI, SCHMID and MÜHLEMANN, 1970). With this index the total surface of the right and left first and second maxillary molars is divided into 20 scoring areas and each area with detectable hard deposits is assigned one scoring unit.

Buffering capacity of diets

The buffering capacity of the phosphate-containing diets was tested in 1:10 diet: water slurries. The suspensions were titrated using an automatic burette connected to a pen writer pH meter◊; pH curves were automatically recorded during gradual addition of 0.1 *n* HCl.

Enzymatic hydrolysis of glycerophosphate

To 2 ml 0.1 M α- or β-sodium glycerophosphate in 0.1 M glycine buffer, 0.0005 M $MgCl_2$, pH 10.5, or to 2 ml 0.1 M α- or β-glycerophosphate in 0.05 M citrate buffer, pH 4.8, 8 ml saline containing 10 mU/ml acid or alkaline phosphatase◊◊ were added. The test solutions were then incubated at 37 °C in a waterbath. After 0, 15, 30 or 60 min 1.0 ml was withdrawn

◊ Potentiograph E 336, Metrohm AG, CH-9100 Herisau, Switzerland

◊◊ Boehringer & Sons GmbH, D-68 Mannheim, Germany

and enzyme activity was stopped by the addition of 10.0 ml 0.0[illegible] N NaOH. The hydrolyzed phosphate was determined by the colorimetric method of FISKE and SUBBAROW (1925). Results were calculated as γ P per ml incubation mixture.

RESULTS

The results are summarized in Tables II, III and IV and in Figure 1.

Growth. The animals on calcium glycerophosphate (Treatment 3) and on calcium chloride (Treatment 9) gained significantly less weight than the animals on all other treatments.

Table IV Enzymatic hydrolysis of sodium glycerophosphate. γ P/ml released after 0, 15, 30 and 60 min incubation at 37 °C of 0.1 M α- or β-glycerophosphate in presence of 10 mU/ml acid or alkaline phosphatase

Incubation time min	Alkaline phosphatase α-isomer	Alkaline phosphatase β-isomer	Acid phosphatase α-isomer	Acid phosphatase β-isomer
0	2.86	2.68	36.12	36.28
15	57.62	40.82	336.49	368.74
30	105.42	101.22	425.37	458.79
60	152.96	166.98	592.35	600.74

Eating frequency. There were significant ($P_F < 0.05$) but small differences in eating frequencies. The animals in the experimental

Table II Average number per rat ($N = 10$) of dentinal fissure lesions (T), advanced fissure lesions (B), carious smooth surface areas (E), calculus scores (CU) and plaque scores (PU). GP = glycerophosphate

Treatments	T	B	E	CU	PU
1 Control	10.4	7.4	6.9	7.9	1.40
2 Control	11.0	8.3	6.0	6.0	0.80
3 25% α-Ca-GP + 75% β-Ca-GP	5.6	1.1	0.0	11.2	1.40
4 25% α-Na-GP + 75% β-Na-GP	7.3	2.1	0.8	9.5	1.40
5 100% α-Na-GP	9.2	4.8	5.5	9.4	1.40
6 100% β-Na-GP	9.1	4.8	1.3	11.0	1.30
7 Na_2HPO_4	9.1	3.3	3.9	15.1	1.90
8 $CaHPO_4 \cdot 2\,H_2O$	8.2	4.6	2.2	11.4	1.60
9 $CaCl_2 \cdot 2\,H_2O$	11.2	8.9	10.6	10.1	1.30
Mean of 10 animals with a standard error $s_{\bar{x}}$ of:	0.7	0.8	1.3	1.5	0.24

12 fissures at risk
20 units at risk
4-grading Index

Table III Eating pattern. Averages of 3 24-hour recordings from 5 rats. Average weight gain over the 21-day experimental period

a) Daily eating time in min
b) Daily eating frequency
c) Daily water intake in ml
d) Daily food intake in g
e) Weight gains in g

Treatments	a	b	c	d	e
1 Control	190	14.7	14.5	8.4	56
2 Control	—	—	—	—	51
3 25% α-Ca-GP + 75% β-Ca-GP	139	15.9	10.5	6.3	31
4 25% α-Na-GP + 75% β-Na-GP	168	17.3	15.7	8.2	48
5 100% α-Na-GP	—	—	—	—	52
6 100% β-Na-GP	—	—	—	—	50
7 Na_2HPO_4	147	17.8	13.3	7.3	49
8 $CaHPO_4 \cdot 2\,H_2O$	142	16.6	12.1	7.9	51
9 $CaCl_2 \cdot 2\,H_2O$	297	16.3	14.3	7.7	32
Mean of 25 determinations on 5 animals (1 determination on 10 animals for weight gains) with a standard error $s_{\bar{x}}$ of:	10	0.6	0.4	0.1	3

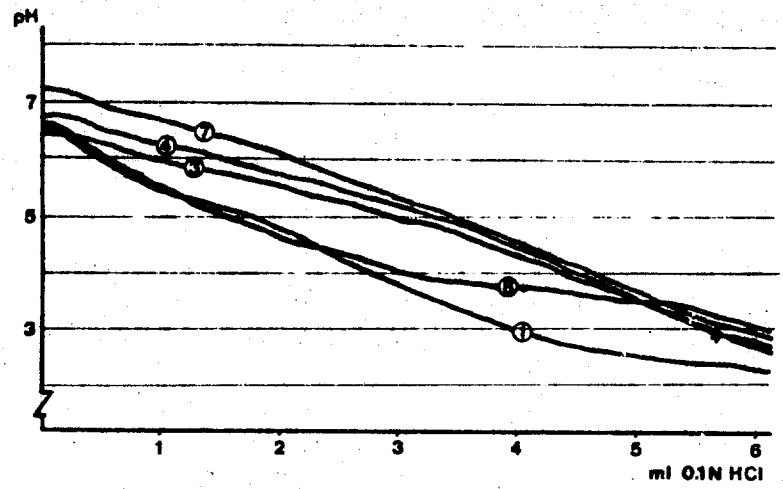

Fig. 1 Buffering capacity of control and phosphate-containing diets. 1:10 diet:water slurries titrated with 0.1 *n* HCl. 1, – (control); 3, calcium glycerophosphate; 4, sodium glycerophosphate; 7, disodium phosphate; 8, dicalcium phosphate

groups ate more frequently than the animals in the registered control group.

Eating time. The animals on calcium chloride (Treatment 9) ate significantly longer than the animals on all other treatments.

Food intake. The diet containing calcium glycerophosphate was eaten less than all other diets.

Water intake. Animals on calcium glycerophosphate and calcium chloride (Treatments 3 and 9) drank less than rats on Treatments 1, 4, 7, 8.

Plaque. There were significant ($P_F < 0.05$) but small differences in plaque formation among treatments.

Calculus. Calculus deposits were more extended in the experimental than in the control groups ($P_F < 0.001$). Glycerophosphate enhanced ($P_F < 0.01$) calculus formation. Disodium phosphate had the maximum calculus-promoting effect.

Smooth surface caries. Caries incidence was reduced by the diets containing β-glycerophosphate ($P_F < 0.001$), while α-glycerophosphate as well as disodium phosphate were ineffective. Dicalcium phosphate was caries inhibitory ($P_F < 0.05$), calcium chloride caries enhancing ($P_F < 0.05$).

Fissure caries. All compounds tested, with exception of calcium chloride, reduced caries incidence at the 0.1% level of significance. There was no difference between α-Na-glycerophosphate and β-Na-glycerophosphate; no difference between dicalcium and disodium phosphate and, again, between α- and β-calcium glycerophosphate and α- and β-sodium glycerophosphate (Treatments 3 and 4). The difference between Treatments 3 plus 4 and 5 plus 6 and between 3 plus 4 and 7 plus 8 were highly significant ($P_F < 0.001$ and < 0.01 respectively).

Buffering capacity of diets. The buffering capacities of diets are shown in Figure 1. Addition of dicalcium phosphate did not enhance the buffer capacity of the diet 2000a, while all other phosphates did.

Enzymatic hydrolysis of α- and β-glycerophosphate. Results are summarized in Table IV. Glycerophosphate was hydrolized much faster by the acid phosphatase than by the alkaline phosphatase. There was no difference in hydrolysis between α- and β-glycerophosphate.

Discussion

Weight gains were strongly impaired in groups receiving calcium chloride or calcium glycerophosphate but were normal in the di-

calcium phosphate group. FORBES (1963) reported that the effect of addition of calcium to the diet of rats was to decrease the growth rate and that this effect disappeared when phosphorus was added to the diet in addition to the calcium. In the present experiment also, growth rate was not impaired when calcium *and* phosphorus were added to the diet as dicalcium phosphate but it was when calcium glycerophosphate was added. This different effect on growth of the 2 compounds indicates that ester-bound phosphate is probably metabolized in a different way than ionic-bound phosphate.

While intake and frequency of eating of cariogenic food were about the same in all experimental groups, water intake was lower in the calcium glycerophosphate and $CaCl_2$ groups. In addition, the eating times were considerably longer in the calcium chloride group. This could explain the higher fissure and smooth surface caries incidence encountered in $CaCl_2$ animals. All phosphates lowered caries incidence. This was probably in part due to a buffering action, as evident from the buffering capacity curves. Moreover, the dicalcium phosphate diet which had a buffer capacity similar to the control diet (because of the low solubility of $CaHPO_4$ at physiologic levels) exerted a considerable cariostatic effect.

The high calculus scores in the glycerophosphate groups indicate that these compounds probably are hydrolyzed by bacterial phosphatases in plaque. The cariostatic effect of the phosphates may hardly have been related to the high calculus incidences: the caries prone calcium chloride group also had high calculus scores.

Smooth surface caries was more strongly inhibited by β-glycerophosphate than by α-glycerophosphate. The reason for this is unknown. *In vitro*, both the α- and β-isomers were hydrolyzed at the same rate by acid or alkaline phosphatase, and not a different rate, e.g. the β-isomer faster as suggested by the smooth surface caries incidence pattern.

Zusammenfassung. Die Wirkungen von Kalzium- oder Natrium-α- oder -β-Glycerophosphat, Dinatriumphosphat, Dikalziumphosphat oder Kalziumchlorid auf Karies, Plaque- und Zahnsteinbildung und Essgewohnheiten wurden in einem 20tägigen Rattentest untersucht. Die Testsubstanzen wurden der kariogenen Diät 2000a in einer 0,6%igen P-Konzentration oder in einer Konzentration aequimolar zu 0,6% P zugesetzt. Glattflächen- und Fissurenkaries wurden durch alle Phosphate gehemmt. Kalziumchlorid-Tiere hatten den höchsten Kariesbefall und die längsten Fresszeiten. Die Testsubstanzen förderten die Plaquebildung unterschiedlich. Alle begünstigten die Zahnsteinbildung. Das Wachstum der Tiere wurde durch Kalziumglycerophosphat und $CaCl_2$ stark gehemmt.

Résumé. Les effets de l'α ou β glycérophosphate de calcium ou sodium, du phosphate disodique, du phosphate de calcium et du chlorure de calcium sur la carie, sur la formation des plaques dentaires et du tartre, ainsi que sur les coutumes de manger et de boire, ont été étudiées chez des jeunes rats pendant 20 jours. Les substances testées furent ajoutées à la diète cariogène 2000a dans une concentration de 0,6% P ou dans une concentration équimolaire à 0,6% P. Tous les phosphates se sont montrés des cariostatiques efficaces. Les rats recevant le chlorure de calcium ont eu l'incidence de la carie la plus haute et ont mangé plus longtemps que les rats de tous les autres groupes. La formation des plaques dentaires a été influencée différemment par les différentes substances. Toutes les substances testées ont favorisé la formation du tartre. Le glycérophosphate et le chlorure de calcium ont eu un effet inhibiteur sur la croissance des jeunes rats.

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CONTENT OF OVER-ALL AND PROTEIN-BOUND CHOLESTEROL IN BLOOD, AND TOTAL CHOLESTEROL AND LIPIDS IN TISSUES DURING LONG-TERM FEEDING OF CALCIUM GLYCEROPHOSPHATE TO RABBITS

L. G. Tertychnaya

It has been noted previously by a group of authors under the leadership of Prof. I. V. Sidorenkov (1-7) that feeding rabbits calcium glycerophosphate over a period of 4-5 months leads to the occurrence of indications of experimental atherosclerosis in its initial phase. Since the methods of obtaining experimental atherosclerosis using calcium glycerophosphate have still been insufficiently studied, we set a goal of investigating the long-term feeding of rabbits with calcium glycerophosphate in doses of 200 mg/1 kg body weight. Such experiments had been done (7), but only for short periods of time.

In accordance with the recommendations of I. V. Sidorenkov, the mechanisms of experimental cholesterol and glycerophosphate atherosclerosis are based on a general mechanism. It leads to the destruction of carbohydrate exchange, leading to an increase in lipid biosynthesis and the use of lipids as energy-giving substances.

It is proposed that in tissue and blood vessels, lipids and cholesterol are in excess and are used as energy-giving substances. In connection with the fact that cholesterol is oxidized more slowly, it will be deposited in the tissues. From this it follows that, in spite of the negation of the role of cholesterol as an etiological factor in atherosclerosis, an important role is ascribed to the latter in this process. Proceeding from this, we have set before us the problem of studying the effect of glycerophosphate on lipid exchange in blood and tissues, as well as the ability of blood protein to bind cholesterol.

A determination was made of the amount of total cholesterol and protein-bound cholesterol, the lipoprotein content in the blood, and the content of total lipids and cholesterol in the tissues.

METHODS OF STUDY

The experiments were conducted on non-strain rabbits weighing 2.5-3 kg. One group of rabbits received a daily preparation of calcium glycerophosphate in food over a period of 5 months (a mixture of alpha and beta isomers) in doses of 200 mg/kg body weight. A second group served as a control, under the same experimental conditions. After the time indicated, the animals were killed with an air embolism, the organs were quickly extracted, and total cholesterol in them was determined by the Liberman-Burchardt color reaction. Total lipid content was determined gravimetrically (8). The amount if lipids is expressed in grams/100 g moist tissue.

Tissues of the heart, liver, kidney, lungs, and skeletal muscles (hip muscle) were subjected to study. In addition, lipoprotein content in the blood was determined using paper electrophoresis according the MacDonald and Berms (9), as well as total lipids, and total protein-bound cholesterol.

RESULTS OF THE STUDY

Twenty rabbits in all were used in the experiment, ten of which served as controls. The results obtained for the determination of total and protein-bound cholesterol in blood serum are presented in Table 1.

TABLE 1. CONTENT OF TOTAL AND PROTEIN-BOUND CHOLESTEROL IN BLOOD SERUM DURING LONG-TERM FEEDING OF CALCIUM GLYCEROPHOSPHATE TO RABBITS

#	Cholesterol content, mg %							
	Control				Experimental			
	Total	Protein bound	Free	% content protein bound	Total	Protein bound	Free	% content protein bound
1	44.0	40,7	3,3	92,5	67,0	33,5	33,5	50,0
2	48,2	43,0	5,2	89,2	77,4	35,4	32,0	45,6
3	69,0	64,0	5,0	92,8	85,0	41,3	43,7	48,5
4	71,8	62,4	9,4	86,9	49,0	25,8	23,2	52,7
5	58,2	51,4	6,5	88,8	45,5	23,5	21,9	51,9
6	59,0	56,1	2,9	95,8	75,4	35,4	40,0	46,9
7	62,0	57,3	4,7	92,4	69,0	32,0	37,0	46,3
8	44,0	39,7	4,3	90,9	79,0	30,2	49,0	40,9
9	47,4	41,5	5,9	87,5	70,0	35,2	34,8	50,2
10	42,3	39,5	13,8	93,3	68,3	32,3	35,0	47,8
M	53,49	49,69	7,1	92,01	68,55	32,46	35,0	44,99
m					±3,9	±1,6	±2,6	±4,79
					P=0,05	P=0,0018	P=0,001	P=0,0004

The results show that long-term supplements of calcium glycerophosphate increases somewhat the content of total blood cholesterol from 53.49 mg % to 68.55 mg % (P = 0.05). In addition, long-term administration of calcium glycerophosphate significantly decreased the content of protein-bound cholesterol from 92.01 % in the control group to 44.99 % in the experimental group. Thus, glycerophosphate would contribute to a release of cholesterol from the protein-cholesterol complexes in the blood.

Next a determination was made of total lipids and beta-lipoproteins in the blood of the control and experimental rabbits (Table 2).

TABLE 2. TOTAL LIPIDS AND LIPOPROTEINS IN BLOOD SERUM OF RABBITS AFTER LONG-TERM FEEDING OF CALCIUM GLYCEROPHOSPHATE

#	Total lipids		Lipoproteins			
	Control	Experimental	Control		Experimental	
			alpha	beta	alpha	beta
1	0,18	0,22	40,3	59,7	57,8	42,2
2	0,30	0,50	58,0	42,0	46,6	53,4
3	0,42	0,48	57,2	42,8	47,3	52,7
4	0,44	0,40	56,0	44,0	34,6	65,4
5	0,34	0,26	69,3	30,7	31,9	68,1
6	0,56	0,42	56,0	44,0	49,6	50,4
7	0,56	0,58	50,0	50,0	40,2	59,8
8	0,32	0,44	65,0	35,0	48,7	51,3
9	0,36	0,46	38,4	62,3	33,5	66,9
10	0,28	0,50	54,5	45,5	44,9	55,1
M	0,37	0,42	54,57	45,53	43,5	56,53
m±		0,17		3,0		2,6
		+13,5				+24,1
P		0,08				0,057

As is evident from the data presented in Table 2, the total lipid content in blood serum has a tendency to increase (13.5 %), although not significantly (P = 0.08).

Beta-lipoproteins also show the same tendency to increase, to 24.1%. The data obtained agree completely with those of Valeyev and Kadanova (6,7)

who used small doses of calcium glycerophosphate.

The next problem was the determination of total lipids in heart and skeletal muscles, and in the tissues of liver, kidney, and lungs. As was already noted above, on the basis of work done by I. V. Sidorenkov and co-workers, with experimental atherosclerosis, there must occur both somewhat of an increase in total lipids (utilized by the tissues as energy-giving substances) and the deposition in the tissues of cholesterol, which is utilised to a lesser degree by the tissues.

Total lipid content in the tissues during long-term introduction of calcium glycerophosphate is presented in Table 3.

TABLE 3. TOTAL LIPID AND CHOLESTEROL CONTENT IN RABBIT TISSUES DURING LONG-TERM FEEDING OF CALCIUM GLYCEROPHOSPHATE (MEAN DATA)

Tiss	# Exper.	Control	Experimental	Change, %	Signif. P
			Total lipids		
Heart	10	3.38	3.38	0	
Liver	10	4.29	5.05	+17.7	0.056
Lungs	10	3.38	4.23	+25.1	0.050
Kidney	10	3.95	4.75	+20.2	0.050
Muscle	10	1.15	1.27	+10.4	0.160
			Cholesterol		
Heart	10	157.1	139.8	−11.1	0.930
Liver	10	326.4	352.4	+ 7.9	0.033
Lungs	10	412.9	437.0	+ 5.8	0.0005
Kidney	10	343.9	386.3	+12.3	0.0001
Muscle	10	74.5	84.0	+12.5	0.113

The results obtained indicate that in the heart and skeletal muscle no significant increase in total lipid level occurs. As regards the other organs (liver, lungs, kidney) a clear ten ency toward an increase is noted in the, although it is only just statistically significant (P = 0.05).

Finally a determination was made of total cholesterol in the same tissues. As is evident from the data presented, only in the tissues of heart and skeletal muscles are practically no changes noted on the part of total cholesterol content. In other tissues, namely in the liver, lungs, and kidneys, a statistically significant increase in cholesterol content is observed.

Thus, the experiments determining total lipids and cholesterol show that calcium glycerophosphate contributes to the deposition of cholesterol in tissues and leads to mobilization of general lipids.

Macroscopically, during long-term introduction of calcium glycerophosphate given over a 5-month period in doses of 200 mg/kg body weight, we did not succeed in noting the appearance of atherosclerotic end-plates in blood vessels, although changes in other organs give a picture of experimental atherosclerosis.

Our investigations therefore confirm that calcium glycerophosphate possesses an atherogenic effect, expressed in a certain increase in blood cholesterol, which aids in its release from protein-cholesterol complexes in the blood and by lipids entering the tissues and the deposition of cholesterol in them.

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СОДЕРЖАНИЕ ОБЩЕГО И СВЯЗАННОГО С БЕЛКАМИ ХОЛЕСТЕРИНА В КРОВИ И ОБЩЕГО ХОЛЕСТЕРИНА И ЛИПИДОВ В ТКАНЯХ ПРИ ДЛИТЕЛЬНОМ КОРМЛЕНИИ КРОЛИКОВ ГЛИЦЕРОФОСФАТОМ КАЛЬЦИЯ

Л. Г. ТЕРТЫЧНАЯ

Ранее коллективом авторов под руководством профессор И. В. Сидоренкова (1—7) было отмечено, что кормление кролико глицерофосфатом кальция в течение 4—5 месяцев приводит появлению признаков экспериментального атеросклероза в на чальной его фазе. Так как методика получения экспериментальног атеросклероза с помощью глицерофосфата кальция еще недоста точно разработана, мы задались целью провести исследования длительным кормлением кроликов глицерофосфатом кальция дозе 200 *мг* на 1 *кг* веса тела. Такие опыты ставились (7), но течение короткого времени.

Согласно представлениям И. В. Сидоренкова, механизм экспе риментального холестеринового атеросклероза и глицерофосфат ный атеросклероз имеют в своей основе общий механизм. Он сво дится к нарушению углеводного обмена, ведущему к повышенно му биосинтезу липидов и их использованию как энергетически веществ.

Предполагается, что в ткани, в том числе и в сосуды, посту пают в избытке липиды и холестерин, которые используются ка энергетические вещества. В связи с тем, что холестерин окисляет ся медленнее, он будет накапливаться в тканях. Из этого вытекает что, несмотря на отрицание роли холестерина, как этиологическо го фактора атеросклероза, последнему отводится важная роль в этом процессе. Исходя из сказанного, мы поставили перед собой за дачу изучить влияние глицерофосфата на обмен липидов в крови и тканях, а также способность белков крови связывать холестерин.

Определению подвергались содержание общего холестерина в

олестерина, связанного с белками крови, содержание липопротеидов в крови и содержание общих липидов и холестерина в тканях.

МЕТОДЫ ИССЛЕДОВАНИЯ

Опыты ставили на беспородных кроликах весом 2,5—3 *кг*. Одной партии кроликов в течение 5 месяцев ежедневно добавляли к корму продажный препарат глицерофосфата кальция (смесь альфа- и бэта-изомеров) в дозе 200 *мг* на 1 *кг* веса тела. Вторая партия кроликов, находившаяся в этих условиях, служила контролем. После указанного срока животных забивали воздушной эмболией, быстро извлекали органы и определяли в них общий холестерин по цветной реакции Либермана-Вурхарда. Содержание общих липидов определяли гравиметрическим методом (8). Количество липидов выражали в граммах на 100 *г* влажной ткани.

Исследованию подвергались ткани сердца, печени, почек, легких и скелетных мышц (бедренная мышца). Кроме того, в крови определяли липопротеиды методом электрофореза на бумаге по Мак Дональд-Бермсу (9), общие липиды, общий и связанный с белками холестерин.

РЕЗУЛЬТАТЫ ИССЛЕДОВАНИЯ

Всего под опытом находилось 20 кроликов, из которых 10 служили контролем. Полученные результаты определения общего и связанного с белками холестерина в сыворотке крови приведены в табл. 1.

Таблица 1

Содержание общего, связанного с белками холестерина в сыворотке крови при длительном кормлении кроликов глицерофосфатом кальция

	Содержание холестерина, *мг* %							
	Контроль				Опыт			
№ п/п	общий	белково-связанный	свободный	%-ное содержание белково-связанного	общий	белково-связанный	свободный	%-ное содержание белково-связанного
1	44,0	40,7	3,3	92,5	67,0	33,5	33,5	50,0
2	48,2	43,0	5,2	89,2	77,4	35,4	32,0	45,6
3	69,0	64,0	5,0	92,8	85,0	41,3	43,7	48,5
4	71,8	62,4	9,4	86,9	49,0	25,8	23,2	52,7
5	58,2	51,4	6,8	88,8	45,5	23,5	21,9	51,9
6	59,0	56,1	2,9	95,8	75,4	35,4	40,0	46,9
7	62,0	57,3	4,7	92,4	69,0	32,0	37,0	46,3
8	44,0	39,7	4,3	90,9	79,0	30,2	49,0	40,9
9	47,4	41,5	5,9	87,5	70,0	35,2	34,8	50,2
10	42,3	39,5	13,8	93,3	68,3	32,3	35,0	47,8
M	53,49	49,69	7,1	92,01	68,55 ±3,9 P=0,05	32,46 ±1,6 P=0,0018	35,0 ±2,6 P=0,001	44,99 ±4,79 P=0,0004

Они показывают, что длительное добавление глицерофосфа кальция несколько повышает содержание общего холестерина крови с 53,49 *мг*% до 68,55 *мг*% (P=0,05). Кроме того, длительн введение глицерофосфата кальция достоверно уменьшает содержание белковосвязанного холестерина с 92,01% в контроле до 44,99% в опыте. Таким образом, глицерофосфат как бы способствует высвобождению холестерина из белково-холестериновых комплексов крови.

Затем производилось определение общих липидов и бэта-липопротеидов в крови контрольных и опытных кроликов (табл. 2).

Таблица

Общие липиды и липопротеиды в сыворотке крови кроликов после длительного кормления глицерофосфатом кальция

№ п/п	Общие липиды		Липопротеиды			
			контроль		опыт	
	контроль	опыт	альфа	бэта	альфа	бэта
1	0,18	0,22	40,3	59,7	57,8	42,2
2	0,30	0,50	58,0	42,0	46,6	53,4
3	0,42	0,48	57,2	42,8	47,3	52,7
4	0,44	0,40	56,0	44,0	34,6	65,4
5	0,34	0,28	69,3	30,7	31,9	68,1
6	0,56	0,42	56,0	44,0	49,6	50,4
7	0,56	0,58	50,0	50,0	40,2	59,8
8	0,32	0,44	65,0	35,0	48,7	51,3
9	0,36	0,46	38,4	62,6	33,5	66,9
10	0,28	0,50	54,5	45,5	44,9	55,1
M	0,37	0,42	54,57	45,53	43,5	56,53
m±		0,17		3,0		2,6
		+13,5				+24,1
P		0,08				0,057

Как видно из данных, приведенных в табл. 2, содержание общих липидов в сыворотке крови имеет тенденцию к увеличению (13,5%), хотя оно и не достоверено (P=0,08).

Такую же тенденцию к увеличению показывают и бэта-липопротеиды, возрастая на 24,1%. Полученные данные полностью совпадают с данными Валеева и Ждановой (6, 7), применявших малые дозы глицерофосфата кальция.

Следующей задачей явилось определение общих липидов в сердечной и скелетной мышцах, в тканях печени, почек, легких. Как уже отмечалось выше, на основании исследований И. В. Сидоренкова и сотрудников, при экспериментальном атеросклерозе должно иметь место некоторое повышение общих липидов в тканях, которые используются ими как энергетические вещества, и отло-

жению в них холестерина, который в меньшей степени используется тканями.

Содержание общих липидов в тканях при длительном введении глицерофосфата кальция приведено в табл. 3.

Таблица 3

Содержание общих липидов и холестерина в тканях кроликов при длительном кормлении глицерофосфатом кальция (средние данные)

Ткань	Кол-во опытов	Контроль	Опыт	Изменения, %	Достовери. P
		Общие липиды			
Сердце	10	3,38	3,38	0	
Печень	10	4,29	5,05	+17,7	0,056
Легкие	10	3,38	4,23	+25,1	0,050
Почка	10	3,95	4,75	+20,2	0,050
Мышца	10	1,15	1,27	+10,4	0,160
		Холестерин			
Сердце	10	157,1	139,8	—11,1	0,930
Печень	10	326,4	352,4	+ 7,9	0,033
Легкие	10	412,9	437,0	+ 5,8	0,0005
Почка	10	343,9	386,3	+12,3	0,0001
Мышца	10	74,5	84,0	+12,5	0,113

Полученные результаты показывают, что в сердечной и скелетной мышцах не происходит заметного повышения уровня общих липидов, что касается других органов — печени, легких, почек, то отмечается явная тенденция к их увеличению, хотя статистически только вероятно (P=0,05).

Наконец производилось определение общего холестерина в тех же тканях. Эти последние результаты приведены в табл. 3. Как видно из приведенных данных, лишь в тканях сердечной и скелетных мышц практически не отмечается изменений со стороны содержания общего холестерина. В других тканях, а именно: в печени, легких, почках наблюдается статистически достоверное повышение содержания холестерина в тканях.

Таким образом, опыты с определением общих липидов и холестерина указывают, что глицерофосфат кальция способствует отложению холестерина в тканях и ведет к мобилизации общих липидов.

Макроскопически при длительном введении глицерофосфата кальция в течение 5 месяцев в дозе 200 *мг* на 1*кг* веса тела не удалось отметить появление атеросклеротических бляшек в сосудах, хотя изменения в других органах дают картину экспериментального атеросклероза,

Наши исследования, таким образом, подтверждают, что глиц рофосфат кальция обладает атерогенным действием, выражащимся в некотором увеличении холестерина в крови, способству освобождению его из белково-холестериновых комплексов кров поступлению липидов в ткани и отложению в них холестерина.

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RESEARCH PAPERS

SODIUM AND CALCIUM GLYCEROPHOSPHATES

A SURVEY

BY J. S. TOAL AND J. I. PHILLIPS

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THE object of this paper is to draw attention to the unsatisfactory position now existing in which commercial supplies of glycerophosphates, although issued as complying with the standards of the British Pharmaceutical Codex, do not actually satisfy the full requirements of the monographs. In order to clarify this position the authors of the present paper produced some pure salts, following factory practice, and investigated them. Published work by other investigators in this subject may be referred to from the bibliography given at the end of the paper[1,2,3,4,5,6,7,8].

DIFFERENTIATION BETWEEN THE ISOMERS

In order to differentiate between the α and β-salts of glycerophosphoric acid an efficient method of assay was required. This was found in the Malaprade reaction of periodic acid on vic. glycols for the estimation of the α-isomer. This method was first applied by Fleury and Paris[9] to the assay of α-glycerophosphates, using arsenious acid for determining the excess of periodic acid, and was later adopted by Pyman and Stevenson[8]. But the present authors found the arsenious acid method somewhat difficult to manipulate, and they prefer the direct procedure of acidifying in the presence of excess of potassium iodide and determining the amount of iodine lost in the reduction of the periodic acid to iodic acid, in comparison with a standard blank. Both methods gave results in complete agreement.

0·3 g. of the crystalline sodium glycerophosphate (or 0·15g. if the α content exceds 50 per cent.) is weighed, placed in a stoppered 250-ml. conical flask and dissolved in a minimum amount of water; 25 ml. of a periodic acid solution (prepared by dissolving 3·674 g. of $Na_3H_2IO_6$ in 37·5 ml. of N sulphuric acid and diluting to 500 ml.) is added, the flask is swirled and set aside for 10 minutes. The reaction that takes place is represented by the equation—

$$\begin{array}{l} CH_2{\cdot}O{\cdot}PO_3Na_2 \\ | \\ CHOH \\ | \\ CH_2OH \end{array} \quad KIO_4 = KIO_3 + \begin{array}{l} CH_2{\cdot}O{\cdot}PO_3Na_2 \\ | \\ CHO \end{array} + HCHO + H_2O$$

After standing, 1 g. of sodium bicarbonate is added to the flask followed by 5 g. of potassium iodide and 10 ml. of dilute hydrochloric acid. The carbon dioxide evolved is allowed to displace the air, the flask is stoppered, and set aside for 10 minutes, and the solution finally titrated with 0·1N sodium thiosulphate. A blank must be carried out in a

similar manner and the difference between the two titrations noted. The reactions that take place on acidification are—

$$KIO_4 + 8HI = KI + 4H_2O + 4I_2$$
$$KIO_3 + 6HI = KI + 3H_2O + 3I_2$$

Thus when 1 molecule of potassium periodate is reduced to potassium iodate a loss of iodine is shown in the final titration.

Hence, from the first equation above, 1 gram-molecule of sodium glycerophosphate is equivalent to 1 gram-molecule of potassium periodate, or 1 gram-molecule of iodine, or 2 litres N/1 thiosulphate. Thus each ml. of 0·1N thiosulphate in the difference noted is equivalent to 0·0162 g. of the α-salt $C_3H_7O_6PNa_2,6H_2O$.

In a mixture of the isomers, the amount of α-sodium glycerophosphate can be found by the above method, and the amount of the β-salt determined by titrating the total glycerophosphate and deducting from that result the titre due to the α-salt. The indicator in the titration should be methyl yellow, which gives a much sharper end point than methyl orange. At this stage of the work it had been assumed that the samples crystallising from the liquors were pure mixtures of α and β-isomers and contained no other titratable matter.

Samples of calcium glycerophosphate were assayed in a similar manner, taking 0·125 g. of the salt, dissolving this directly in 25 ml. of the periodic solution and completing the titration in the manner already described; the difference between the titration of the blank and the test being recorded, 1 ml. of 0·1N thiosulphate being equivalent to 0·0105 g. of anhydrous calcium glycerophosphate.

When analysing sodium glycerophosphate liquors from the original combination, and also the mother liquors from which crops of crystals have been taken, it is essential that any free glycerin shall be completely removed, by repeated extraction with alcohol, because even traces of glycerin give rise to error in the assay for the α-salt.

Preparation of Pure α and β-Sodium Glycerophosphate

Crude sodium glycerophosphate was prepared by combining, under vacuum, two equivalents of glycerin with one equivalent of sodium acid phosphate and then hydrolysing the resultant diglyceryl ester with sodium hydroxide. The glycerin was removed by extraction with alcohol and, after separation, the alcohol that remained was expelled by evaporation. Samples from three bulk batches were adjusted to contain 50 per cent. w/w of $C_3H_7O_6PNa_2 5\frac{1}{2}H_2O$ and the solution analysed. The results are shown in Table I:

TABLE I

Batch	Sp. gr. at 15·5°C.	Assay by titration	Assay by ignition	Residue at 150°C.	α-isomer ($6H_2O$)
		per cent.	per cent.	per cent.	per cent.
A	1·274	50·0	50·7	34·4 (theory 34·3)	23·15
B	1·277	49·5	50·6	35·4	21·0
C	1·277	50·0	50·7	36·3	22·0

These figures show the general constancy of the combination.

The bulked solution of these three batches was evaporated and adjusted to a strength of 70 per cent. w/w of $C_3H_7O_6PNa_2,5\frac{1}{2}H_2O$ and allowed to crystallise during several days. The crystals were removed and washed with alcohol. The mother liquor was again set to obtain further crystals and this procedure repeated until six different crops had been collected. The liquor from the sixth crop was uncrystallisable.

These six crops of crystals, after washing with alcohol to ensure the removal of any adherent glycerin, were assayed for their α β-content. The results are shown in Table II.

TABLE II

Crop	α-salt ($6H_2O$)	β-salt ($5H_2O$)	Total
	per cent.	per cent.	per cent.
1 ...	13·9	85·2	99·1
2 ...	71·0	30·9	101·9
3 ...	91·5	7·85	99·35
4 ...	28·5	72·9	101·4
5 ...	71·0	30·1	101·1
6 ...	42·0	57·8	99·8

Each of the six samples satisfied all the chemical requirements of the B.P.C., but all failed to comply with the opening definition that " sodium glycerophosphate is the sodium salt of β-glycerophosphoric acid."

Table II shows that the first crop of crystals contain the highest proportion of the β-isomer, whereas the third crop consisted almost entirely of the α-variety. These two crops of crystals were taken separately and twice recrystallised; finally separated from their mother liquor by centrifuging then washed with alcohol and dried at 60°C.

The analysis of these recrystallised preparations (given in Table III) shows that two pure isomers were obtained, the α-isomer containing 6 molecules of water of hydration and the β-isomer 5 molecules.

TABLE III

Tests	1st crop (twice recrystallised) β-isomer	3rd crop (twice recrystallised) α-isomer
	per cent.	per cent.
Loss at 150°C. ...	29·75 (theory 29·42)	33·25 (theory 33·34)
Assay (titration) ...	99·30 $C_3H_7O_6PNa_2,5H_2O$	99·50 $C_3H_7O_6PNa_2,6H_2O$
Assay (gravimetric) ...	99·20 " "	99·20 " "
α-isomer ...	absent	99·00 " "
Sp. gr. of 50 per cent. w/w solution ...	—	1·271 at 15·5°C.

A saturated solution of the α-isomer contained the equivalent of 63·5 per cent. w/w of $C_3H_7O_6PNa_2,6H_2O$ and a saturated solution of the β-isomer contained 45·5 per cent. w/w of $C_3H_7O_6PNa_2,5H_2O$.

The crystals of the α-isomer were hard and of a semitransparent nature, quite distinct from those of the β-isomer, which were fragile and of needle-like appearance.

The uncrystallisable liquor, which represented 10 per cent. of the original combination, was diluted with sufficient water to keep any α and β-calcium glycerophosphate in solution and then treated with a solution of calcium acetate. The precipitate that formed was collected

and analysis indicated its being a di-ester $CHOH(CH_2.O.PO_3Ca)_2$; the figures based on material dried at 150°C. are given in Table IV.

This calcium salt did not reduce periodic acid solution, and therefore was not a vic. glycol. An approximate computation showed that 100 g. of presumptive sodium glycerophosphate in the uncrystallisable mother

TABLE IV

	Calcium	Residue on ignition	Phosphorus
	per cent.	per cent.	per cent.
For suggested formula	24·4	77·5	18·90
For the precipitate	25·5	78·1	18·60

liquor consisted of 55 parts of di-ester and 45 parts of true sodium glycerophosphate; yet, when adjusted to a liquor containing 50 per cent. of apparent sodium glycerophosphate ($5\frac{1}{2}H_2O$) as estimated by titration, it agreed with the standards set in the B.P.C.

Calcium Glycerophosphate

Calcium salts were prepared from solutions of the pure α and β-salts of sodium glycerophosphate.

With the β-salt, the method employed was that of treating the solution with an excess of 30 per cent. w/v aqueous solution of calcium acetate, and following with an excess of alcohol sufficient to throw down the calcium glycerophosphate. This was collected, washed free from calcium and sodium acetates by means of alcohol, dried and then examined. It held one molecule of water of hydration ($1H_2O$) and was soluble to the extent of 0·96 g. of anhydrous material in 100 ml. of solution at room temperature.

With the α-salt, two methods of preparation were employed: one precisely as just described for the β-salt; the other, that of using more concentrated solutions (without the use of alcohol) and allowing the calcium glycerophosphate to crystallise from the supersaturated condition. In both cases the material was collected, washed with alcohol, dried and analysed. The precipitate effected by means of alcohol contained 2 molecules of water of hydration ($2H_2O$), and that depositing from the supersaturated solution only 1 molecule ($1H_2O$). The analytical figures for the 3 salts are given in Table V.

Solubility of the Two α-Salts in Water

Each salt was treated with distilled water, leaving a little of the salt undissolved. After about an hour's digestion 50 ml. of solution was withdrawn and titrated and the amount of calcium glycerophosphate in solution calculated. The solution of the $2H_2O$ salt contained 4·3 per cent. of the anhydrous salt and that of the $1H_2O$ salt 1·17 per cent. The suspensions were set aside for 3 days, with occasional shaking, and then again tested. The amount of $2H_2O$ salt in solution had dropped to 4·0 per cent., whilst that of the $1H_2O$ salt remained at 1·17 per cent. The

mixture containing the $2H_2O$ salt was now tested periodically until constant solubility was attained. This occurred at 1·26 per cent. of the anhydrous material after about 4 weeks' digestion at from 20° to 25°C.; considerable precipitation having taken place. The residuum of this digest was collected, washed with alcohol, dried and examined: it consisted of α-calcium glycerophosphate now associated with only 1 molecule of water of hydration. Thus a change in constitution has occurred, and the more soluble but unstable salt with 2 molecules of water has reverted to the less soluble but stable salt with 1 molecule of water.

These 2 α-hydrates were rendered anhydrous: the $2H_2O$ salt lost all the water at 140°C., but the $1H_2O$ salt had to be dried at 150°C. On exposing the anhydrous salts to the atmosphere, both absorbed moisture and returned to their original states of hydration, that is to say, to the $2H_2O$ and $1H_2O$ hydrates, and dissolved 1 in 25 and 1 in 100 of water respectively, just as they did before subjection to dehydration. Thus there are 2 hydrates chemically the same, but having different solubilities. Possibly, the structures of the two hydrates differ, and a different arrangement of the calcium and glycerophosphate ions and of the molecules of water in the lattices could explain the effects noted.

X-ray diffraction data of the two hydrates and of the corresponding dehydrated salts are given below.

TABLE V

X-RAY DIFFRACTION DATA OF THE TWO HYDRATES OF α-CALCIUM GLYCEROPHOSPHATE, INTERPLANAR SPACINGS IN Å WITH APPROXIMATE RELATIVE INTENSITIES

Monohydrate	Dehydrated Monohydrate	Dihydrate	Dehydrated Dihydrate
13·0 vs.	14·8 vvs.	14·2 vs.	10·9 vvs. broad line
6·7 w.	about 4·0 s. diffuse band	7·1 mw.	4·19 mw.
about 4·0 w. diffuse band			
5·5 mw.	2·73 w.	5·17 m.	3·86 ms.
4·64 s.	1·99 w.	3·49 m.	3·48 ms.
3·34 mw.		2·22 vw.	3·27 w.
2·89 m.		2·05 w.	3·04 mw.
2·75 w.		1·81 w.	2·57 w. broad
2·33 w.		1·72 w.	2·11 w. broad
2·10 vw.			1·85 vw.
1·83 w.			1·73 vw.

s Strong. m Medium. w Weak.

These experimental data demonstrate the distinct structural difference of the two hydrates and of their respective dehydrated salts. It is interesting to compare the effect of dehydration on the diffraction patterns, and in particular on the strong high spacing lines, in the 2 cases. For the dehydrated monohydrate the pattern is almost non-existent except for a strong diffuse band and an extremely strong line of higher spacing than the maximum spacing line of the original monohydrate. This suggests that the water molecules are important in holding the calcium glycerophosphate units of the structure together, and that on their removal these units drift apart and the structure becomes much less ordered. On the other hand, the dehydrated dihydrate has a good strong pattern with a decrease in spacing of the maximum spacing line from that of the original dihydrate. This suggests that on removal of the water mole-

cules the remaining calcium glycerophosphate units can pack together in a highly ordered crystal structure which can, however, readily revert to that of the original hydrate.

COMMERCIAL SAMPLES OF CALCIUM GLYCEROPHOSPHATE

Samples of this salt were obtained from the principal manufacturers and subjected to the B.P.C. tests: the results are given in Table IV. The last three samples in the table were prepared by the present authors.

TABLE VI

ANALYTICAL DATA OF SAMPLES OF CALCIUM GLYCEROPHOSPHATE

Sample	Loss at 150 C.	Assay by titration	Assay by ignition	α-isomer	Solution 1 in 50
	per cent.	per cent	per cent.	per cent	
A	7·6	93·0	99·46	79·76	Soluble, but flocculent. Precipitate overnight.
B	10·8	94·60	98·80	78·92	Soluble but within 1 hour commenced to flocculate.
C	7·5	94·18	101·16	79·64	Not complete; also commenced to flocculate on standing.
D	10·5	93·90	97·85	51·91	Soluble; flocculated on standing
α-salt $2H_2O$	13·9	98·90	99·63	99·15	Soluble; only very slight flocculation overnight.
α-salt $1H_2O$	7·0	99·33	99·5	100·90	Not complete but dissolves quickly 1 in 120 to a clear solution.
β-salt $1H_2O$	9·8	100·22	100·0	absent	Not complete but dissolves slowly 1 in 120.
B.P.C. Standards	Not more than 15·0	94·50	98·0		Dissolves with slight turbidity

All figures are based on material dried at 150 C.; B.P.C. directs drying at 130 C.

Reference to Table VI will show that 3 out of the 4 samples analysed failed to meet the standard set for titratable matter, and yet reached the requirement (or practically so) as regards ash. This at once brings up the question as to whether the preparations or the standards are wrong; and when the different solubilities are also taken into account, emphasis is added to the question. It is clear that there are some difficulties in manufacture, for otherwise there would be greater uniformity in the products, and it may be asked whether the requirements of medicine are such that more precise standards, to secure a closer approach to the pure salt, should be set up or whether such differentiation is of little therapeutic importance and wider limits should be allowed: such wider limits being, of course, rather arbitrary and merely arranged to suit good commercial practice. But on the question of solubility there is room for debate: insisting upon a degree of solubility which is not a stable property of the salt serves no useful purpose, but rather the reverse. If a pharmacist looks to a salt being soluble 1 in 50 of water and then, when using it in compounding, finds precipitation takes place, he is perturbed and at a loss; but if he knows that an ultimately stable solution can only exist at 1 in 100 some of his difficulties will not arise. Again, if there is no sound therapeutic reason for preferring one or other of the isomers, then degree of solubility in water is of still less importance since both salts will be readily soluble in the acid gastric juice.

PROPOSED CHANGES TO THE B.P.C. MONOGRAPHS FOR SODIUM AND CALCIUM GLYCEROPHOSPHATE

Resulting from their present investigation the authors suggest that the following changes might be made in the B.P.C. monographs.

Sodium Glycerophosphate

1. To omit the chemical formula and also the description referring to the material as "the sodium salt of β-glycerophosphoric acid." Instead, the description to be "Sodium glycerophosphate may be the sodium salt of α-glycerophosphoric acid $C_3H_7O_6PNa_2.6H_2O$ or the sodium salt of β-glycerophosphoric acid $C_3H_7O_6PNa_2.5H_2O$ or any mixture of these isomers."

2. The assay to remain as at present, but referred to the material dried at 150°C. Standards to be altered to "Not less than 98 per cent. and not more than 102 per cent. of $C_3H_7O_6PNa_2$ calculated on the material dried at 150°C."

3. Loss at 150°C. not to exceed 34·5 per cent. This admits the hexahydrate with 33·34 per cent. of water and allows a little additional moisture.

Sodium Glycerophosphate 50 per cent. Liquor

1. To be described as "An aqueous solution containing about 50 per cent. w/w of α-sodium glycerophosphate hexahydrate, or a mixture of the α and β-isomers."

2. The assay to refer to the equivalent of anhydrous sodium glycerophosphate in the liquor. The limits to be 32·6 per cent. and 36·0 per cent. of $C_3H_7O_6PNa_2$.

These limiting figures are derived from the following considerations —50 per cent. of the hexahydrate is represented by 33·6 per cent. of anhydrous salt, which becomes 32·6 per cent. when allowing for a purity limit of 98 per cent. Similarly 50 per cent. of the pentahydrate is equivalent to 35·3 per cent. of anhydrous salt, and this, calculated to the upper limit of 102 per cent. allowed for the crystalline salt, becomes 36·0 per cent.

3. The specific gravity range to be from 1·255 to 1·300. This covers the two limits of the assay and also allows for the presence of 2 per cent. of glycerin.

All the other present limits, including that for glycerin, could remain since no difficulty has been experienced in meeting these standards.

Calcium Glycerophosphate (Dihydrate)... Now included in the B.P.C.

1. The loss on drying to be determined at 150°C.

2. The assay should be the method of Bennett and Campbell[10] in which igniting with ammonium nitrate and reigniting with nitric acid is adopted. The present authors found this method very satisfactory.

3. 1 g. of the salt should dissolve in 50 ml. of water at a temperature below 20°C. within a few minutes. On further dilution to 100 ml. and standing overnight no more than a very slight precipitate should develop.

The test for titratable matter and other limiting tests allow ample margin for the manufacturers' difficulties in production.

Calcium Glycerophosphate (Monohydrate)... Not recognised in the B.P.C.

1. It is recommended that official recognition be given to both α and β-salts both approximating to the monohydrates.

These are the salts which are stable in water, and which satisfy all the official requirements at present set out for the dihydrate with the single exception of solubility; the present requirement being that of an unstable condition.

1 g. of the salt should dissolve in 130 ml. of water within a few minutes. Both salts will dissolve in a smaller quantity of water, but the rate of dissolution is slow with the β-salt.

Summary

1. A method has been described for the determination of the α-isomer of glycerophosphoric acid.

2. The preparation and properties of pure crystalline α- and β-sodium glycerophosphate have been described.

3. The isolation of "an impurity," occurring from a side reaction in the primary combination and which seems to approximate to a di-ester, is reported.

4. Two types of α-calcium glycerophosphate have been prepared: a dihydrate which is unstable in water and a monohydrate which is stable.

5. The properties of the two hydrates of the calcium salt have been investigated and differences in their physical behaviour are reported.

6. The preparation and properties of β-calcium glycerophosphate have been described.

7. Commercial samples of calcium glycerophosphate have been examined and their departure from official standards noted.

8. Suggestions for the official monographs have been made.

Special thanks from the authors are due to Dr. D. E. Palin, of Imperial Chemical Industries, Limited, Research Department, Widnes, for his interest in the crystal structure and for discussion of the X-ray diffraction data which he supplied.

We also wish to acknowledge the enthusiastic help of our technician, Mr. W. Rone, whose work made much of this paper possible, and we thank the Directors of Evans Medical Supplies, Limited, for permission to publish our work.

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DISCUSSION

The paper was presented by Mr. J. S. Toal.

MR. G. J. W. FERRY (Manchester) said that 23 years ago it was shown that the calcium glycerophosphate then on the market was the monohydrate, and not the dihydrate which was introduced into the Codex later. The 50 per cent. solution of sodium glycerophosphate was standardised by some makers on anhydrous sodium glycerophosphate and by others on the basis of the salt containing 5½ molecules of water of crystallisation, while others varied between those two limits. There was very little diester in some makes. The insolubility of calcium glycerophosphate had been a difficulty in making the compound syrup. He wondered whether it would not be best to leave out the calcium glycerophosphate altogether and put in some soluble form of phosphoric acid; the main object of putting glycerophosphate in was to get the tonic effect of the phosphorus, and they could very well dispense with the calcium. He asked whether there was any difference in therapeutic activity between the α-and β-glycerophosphoric acids, and also whether there was any evidence to show that glycerophosphoric acid and its salts had any therapeutic action which was not possessed by phosphoric acid and its salts.

DR. G. E. FOSTER (Dartford) asked for information about magnesium glycerophosphate. This formerly came from Germany and usually complied with the requirements of the British Pharmaceutical Codex. After the war, however, it had been difficult to get any of British manufacture which complied.

DR. K. BULLOCK (Manchester) criticised the author's statement that the solubility of one of the isomers was not of great importance, because in any case it would dissolve in the acid gastric juice. Surely the salt would be reprecipitated again in the intestine; few substances were absorbed from the stomach. If, on the other hand, it was a real decomposition, why should not the separate products be given, instead of combining them and then allowing them to disintegrate again in the stomach?

MR. R. W. GILLHAM (Leeds) asked whether the authors considered that their work had thrown any light on the problem of the precipitation which occurred in samples of compound syrup of glycerophosphates.

MR. J. S. TOAL, in reply, referring to the calcium salt, said that one could get the dihydrate only by precipitation with alcohol. The two hydrates had different solubilities. The dihydrate would dissolve with a 1 in 20 initial solubility, and a stable solubility of 1 in 100. The monohydrate had an initial solubility of 1 in 100. They had found that 50 per cent. solutions were low in diester. He suggested that the formula for the compound syrup of glycerophosphates ought to be based on the pure salts; as it was now each manufacturer had a different preparation, and they did not comply with the B.P.C. With one or two exceptions, none of the magnesium salts on the market to-day would pass the Codex tests. The standard required 97 per cent. by ignition and 95 per cent. by titration, but many of them were less than 90 per cent.

Arch. inst. farmacol. exptl. med. 2:48-58. 1950.

Higher Council of Scientific Investigations

Institute of Experimental Pharmacology-Madrid Section

Action of sodium β-glycerophosphate in blood sugar and as modifier of the action of insulin

by Doctors J.M. Torres-Acero Fernandez and F. Andolz Aguilar

The new channels in the pharmacology of β-glycerophosphate opened by Professor Velazquez and his school, by making known its spasmolytic actions on uterine fiber (1),(2),(3) and stimulant of the respiratory center (4), were the motives which induced us to study the behavior of this drug with respect to blood sugar, as well as the modifications that it imparts to insulin hypoglycemia.

The metabolic role of sodium β-glycerophosphate, already amply discussed in the works mentioned, is in part confirmed by our current study, and permits us to derive some new applications of the same which may have clinical interest, its possible use in the prevention of the nervous accidents of insulin therapy. To this effect adenosine triphosphoric acid (ATP), with like metabolic action, has already been recommended for this same purpose and confirmed in a psychiatric clinic by Berenstein and Levi (5).

Method

Adult rabbits, without regard to sex, were used. They were subjected beforehand to a twenty-four hour fast. Blood was obtained from them by section of the marginal vein of the ear. Blood samples were taken at thirty minute intervals, after the administration of β-glycerophosphate or insulin sc.

Blood glucose was determined by the Folin-Malmros micromethod, because of the advantage that only 0.1 cubic centemeter of blood is supposed to be used, while almost twenty blood samples had to be taken from each animal in one day.

The Folin-Malmros method is as follows: 0.1 cc of whole blood, taken with a suitable pipette of 0.1 cc total capacity, are added to 10 cc of dilute tungstic acid, washing the pipette in the same two or three times; it is mixed and after fifteen minutes of settling the transparent liquid is separated by filtration.

In a 25 cc Folin-Wu tube, 4 cc of the transparent liquid are placed and 2cc of potassium ferricyanide and 1 cc of cyanide alkaline solution are added. The tube is subjected to a boiling water bath for eight minutes and then is cooled in a cold water bath.

5 cc of the phosphoferric Ghatti gum mixture are added to it and allowed to settle for two minutes. The tube is then filled up to 25 cc with distilled water; it is mixed and after ten minutes a photocolormetric reading is made, using a green filter and lining up as zero concentration solution, adjusted to 100% of transmission, a Folin-Wu tube with 4 cc of distilled water, with the one which was handled in the same way as with the control tubes.

The standard curve is constructed by preparing glucose samples of different concentrations, which are tolerated in the way indicated above to determine the percentage of transmission corresponding to each known concentration.

Preparation of the reagents is as follows:

Dilute tunstic acid. To a measured flask of 250 cc containing some 200 cc of water are added 5 cc of sodium tungstate at 10%; after mixing 10 cubic centimenters of 2/3 normal sulphuric acid are added slowly, shaking again and filling up with water to 250 cc. This solution must be renewed frequently.

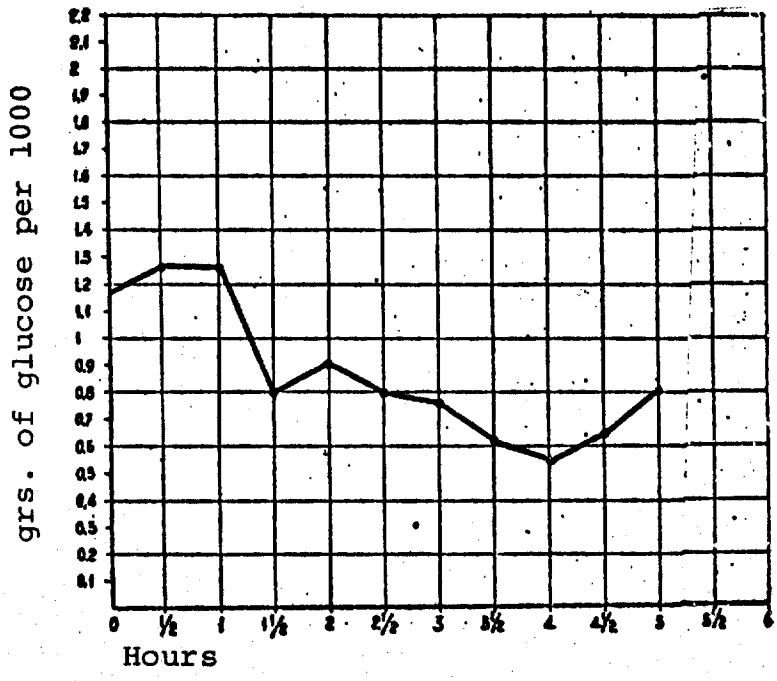
Watery solution of potassium ferricyanide at 0.4%, kept in an amber colored bottle and renewed frequently.

Sodium cyanide alkaline solution. To 160 cc of anhydrous sodium carbonate at 10% are added 300 cubic centimeters of a recently prepared solution of sodium cyanide at 1%, filling with distilled water to 1,000 cubic centimeters. One must be extremely careful in the handling of such a toxic solution.

Phosphoferric Ghatti gum solution. A sack of loosely woven cloth containing 20 grams of Ghatti gum is submerged in a liter of distilled water, and is kept near the surface; at 18 hours it is filtered through plushy material while collecting all the filtered liquid, to which are added five grams of ferric sulphate dissolved in 75 cc of phosphoric acid at 85% and 100 cc of distilled water. 20 cc of potassium permanganate at 1% are added, and it is maintained at 37° for forty-eight hours, then being ready for use.

Graph 1. Rabbit no. 4, weighing 2,500 kilograms. Sc. injection of 0.50 grams of sodium β-glycerophosphate (0.057 grams of organic P.) per kilo of weight. Values of the glycemia: Fasting, 1.18 grams per 1,000; at 30 minutes after injection of the glycerophosphate, 1.27; at 60 minutes, 1.27,1.17; at 90 minutes, 0.80; at 120 minutes, 0.91; at 150 minutes, o.80; at 180 minutes, 0.77; at 210 minutes, 0.62; at 240 minutes, 0.55; at 270 minutes, 0.64; at 300 minutes, 0.82.

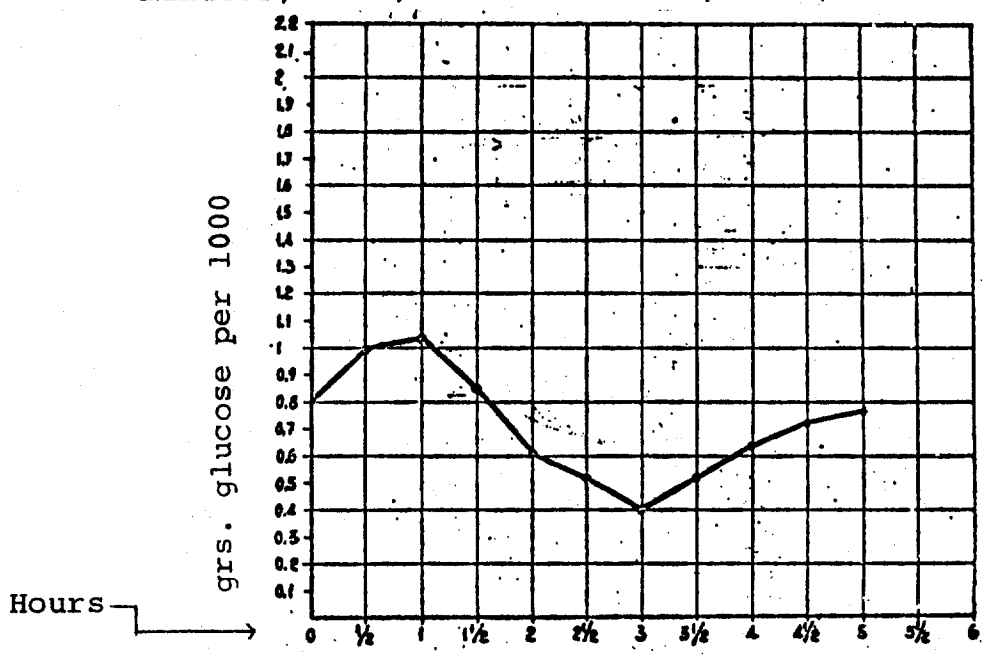
Graph 1 follows on page three.



Results

Effects of sodium β-glycerophosphate on glycemia. The sodium β-glycerophosphate acts on the glycemia producing a clear descent, after a slight increase of the same. The effect is maintained for three or four hours, regaining the level of glycemia at the end of this time at its initial value (glycemia while fasting).

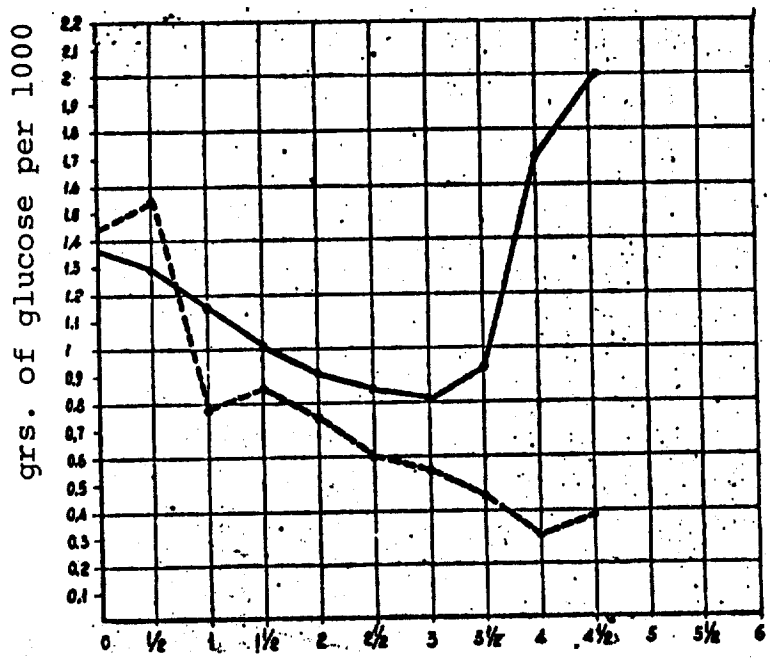
Graph 2. Rabbit no. 5, weighing 1.800 kilograms. Sc. injection of 0.50 grams of sodium β-glycerophosphate (0.057 grams of organic P.) per kilo of weight. Values of the glycemia: Fasting, 0.80 grams per 1,000; at 30 minutes from injection with the glycerophosphate, 1.00; at 60 minutes,1.03; at 90 minutes, 0.85; at 120 minutes, 0.60; at 150 minutes, 0.53; at 180 minutes, 0.40; at 210 minutes, 0.51; at 240 minutes, 0.63; at 270 minutes, 0.71; at 300 minutes,0.75.



The lowest values of glycemia reached were in the range of 0.50 to 0.40 grams per 1,000, without presentation in either case in the rabbits of a convulsive picture.

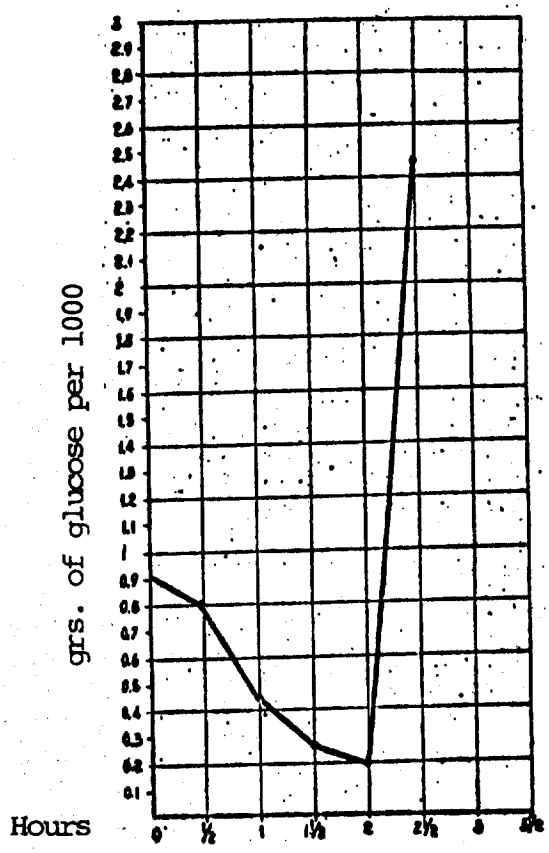
In graphs nos. 1 and 2, we show the glycemia curves obtained in rabbits 4 and 5, which were injected sc. with sodium β-glycerophosphate, at a rate of 0.50 grams per kilogram of weight, equivalent to 0.057 grams of organic P.

Graph 3. Rabbit no. 7, weighing 2.350 kilograms. Sc. injection of 2 units of insulin per kilogram of weight. Values of the glycemia: Fasting, 1.35 grams per 1,000; at 30 minutes from injection with insulin, 1.30; at 60 minutes, 1.15; at 90 minutes, 1.00; at 120 minutes, 0.90; at 150 minutes, 0.85; at 180 minutes, 0.81; at 210 minutes, 0.92; at 240 minutes, 1.70; at 270 minutes, 2.01. (black curve). The same rabbit. Sc. injection of 2 units of insulin per kilogram of weight, plus 0.50 grams of sodium β-glycerophosphate per kilogram of weight. Values of the glycemia: Fasting, 1.43 per 1,000; at 30 minutes from injection with glycerophosphate and insulin, 1.55; at 60 minutes, 0.77; at 90 minutes, 0.86; at 120 minutes, 0.75; at 150 minutes, 0.60; at 180 minutes, 0.55; at 210 minutes, 0.46; at 240 minutes, 0.36; at 270 minutes, 0.38. (red curve).



Graph 4. Rabbit no. 9, weighing 2.150 kilograms. Sc. injection with 2 units of insulin plus 0.75 grams of sodium β-glycerophosphate per kilo of weight. At 150 minutes injection was given with 0.50 grams of glucose per kilo. Values of the glycemia: Fasting. 0.91 grams per 1,000; at 30 min. from injection with glycerophosphate and insulin, 0.80; at 60 min.,0.45; at 90 min., 0.25; at 120 min., 0.19; at 150 min., 2.45.

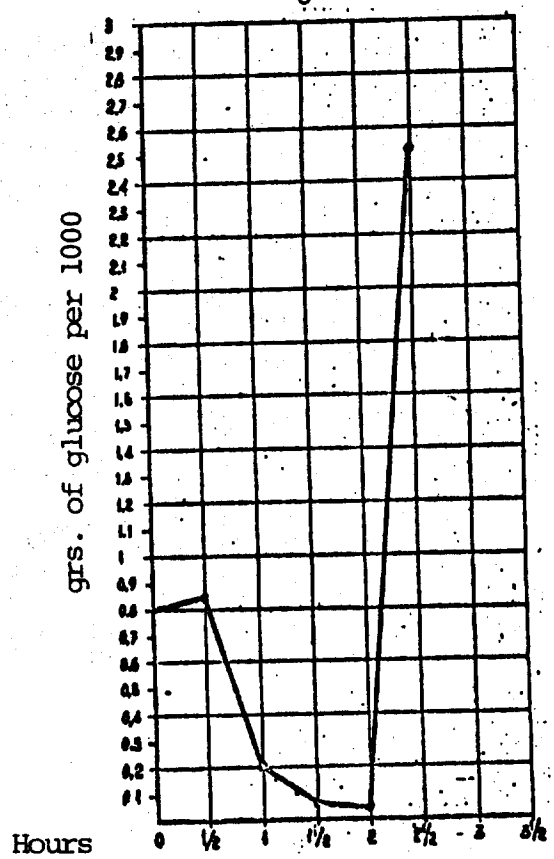
Graph 4.



Graph 5. Rabbit no. 10, weighing 1.750 kilograms. Sc. injection with 2 units of insulin plus 0.75 grams of sodium β-glycerophosphate per kilogram of weight. At 150 minutes, 0.50 grams of glucose per kilo was injected. Values of the glycemia: Fasting, 0.80 grams per 1,000; at 30 minutes from injection with glycerophosphate and insulin, 0.84; at 60 minutes, 0.21; at 90 minutes, 0.06; at 120 minutes, 0.05; at 150 minutes, 2.50.

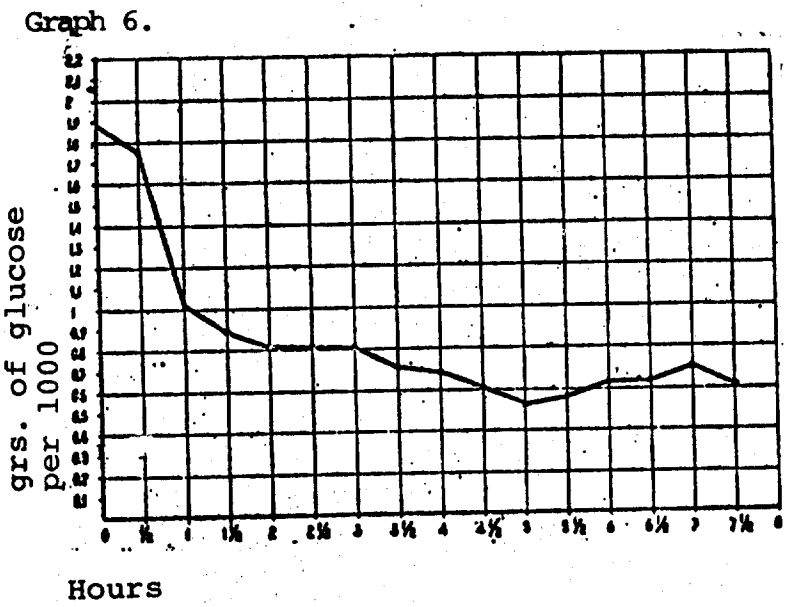
Graph 5 will follow on page 6.

Graph 5.



Effects of the association sodium β-glycerophosphate-insulin on glycemia. On using insulin with β-glycerophosphate we obtain a very marked descent of glycemia, arriving at very low levels of the latter. By comparing these results with those obtained with insulin alone, it is clearly observed that the greatest and longest descent corresponds to the administration of sodium β-glycerophosphate with insulin.

Graph 6.



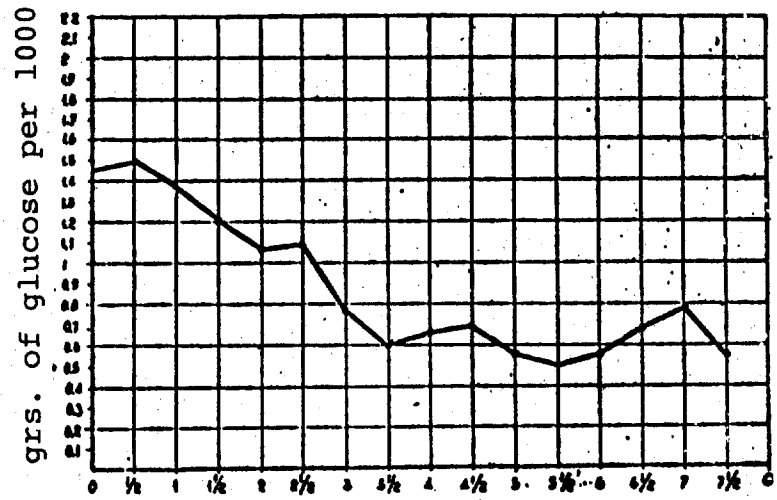
Rabbit nc. 11, weighing 2.250 grams. Sc. injection with 2 units of insulin, plus 0.50 grams of sodium β-glycerophosphate per kilo of weight. Values of the glycemia: Fasting: 1.87 gr. per 1000; at 30 min. from injection with glycerophosphate and insulin, 1.75; at 60 min.,1.01; at 90 min., 0.83; at 120 min., 0.82; at 150 min., 0.81; at 180 min., 0.80; at 210 min., 0.71; at 240 min., 0.68; at 270 min., 0.60; at 300 min., 0.52; at 330 min., 0.55; at 360 min., 0.63; at 390 min., 0.63; at 420 min., 0.68; at 450 min., 0.60.

In graph no. 3 we represent the glycemia curves obtained in one rabbit, one by the administration of two units of insulin per kilogram of weight (black curve), and the other by the administration of insulin in the same dose, and sodium β-glycerophosphate, 0.50 grams per kilogram of weight (red curve).

This experiment, in its second phase, was carried out when the rabbit was already totally recovered.

In graphs nos. 4 and 5, we show the glycemia curves obtained with rabbits nos. 9 and 10, injected with a somewhat larger dose of sodium β-glycerophosphate (0.75 grams per kilo of weight). In these graphs we can clearly appreciate the very low levels of glycemia reached on administering insulin and β-glycerophosphate, In these two cases, it was necessary to use glucose for them to recover from the hypoglycemic coma produced. This recovery was very easily effected by means of the sc. injection of 0.50 grams of glucose per kilogram of weight.

Graph 7. Rabbit no. 14, weighing 2.000 kilograms. Sc. injection of 2 units of insulin, plus 1.25 milligrams of sodium adenosine triphosphate (ATP)(0.045 gr. of organic P.) per kilogram of weight. Values of the glycemia: Fasting: 1.44 per 1000; at 30 min. from injection with ATP and insulin, 1.48; at 60 min., 1.37; at 90 min., 1.20; at 120 min., 1.06; at 150 min., 1.07; at 180 min., 0.77; at 210 min., 0.60; at 240 min., 0.65; at 270 min., 0.68; at 300 min., 0.55; at 330 min., 0.50; at 360 min., 0.55; at 390 min., 0.68; at 420 min., 0.77; at 450 min., 0.55.



We represent graph no. 6 to demonstrate that the hypoglycemic-like effect is of long duration. In this experiment the glycemia continued at low levels eight hours after the administration of sodium β-glycerophosphate.

We shall also point out that on using sodium β-glycerophosphate with insulin, the initial slight hypoglycemic-like effect that we indicate by the use of β-glycerophosphate alone either does not appear, or if it does, it is in an almost imperceptible way (graphs numbers 3,4,5, and 6).

As we have already indicated previously, results obtained with sodium β-glycerophospahte are very similar to those presented by using ATP; graph no. 7 represents the glycemic curve by administration of ATP with a dose of 1.25 milligrams per kilogram of weight (equivalent to 0.045 grams of organic P) plus insulin with a dose of two units per kilogram of weight.

Discussion

The results we obtained on the glycemia by the administration of sodium β-glycerophosphate must be attributed, without any doubt, to the important role this substance possesses in the metabolism of carbohydrates; let us recall the interest that glycerophosphate acid has the Meyerhof cycle.

The fact that, at the present time, triphosphoric acid, according to the recent works of Cori and collaborators (6), is considered necessary together with the hexoquinase enzyme in the action mechanism of insulin, leads us to think that sodium β-glycerophosphate would act likewise, facilitating the transformation of glucose into glucose-6-phosphate (Robinson ester).

The sodium β-glycerophosphate would be responsible for this transformation, since as phosphoric ester, it would act as transmitter or mediator of phosphoric acid, being, then, an activator of the substrata of glycolysis. We consider, therefore, as indispensible in this action of β-glycerophosphate to the phosphatized fraction [sic]. Our supposition is corroborated by the works of Franks and collaborators (7),(8), which recommend the use of sodium phosphate in a diabetic coma, since they have demonstrated the existence of a deficit of phosphates in this syndrome, since a rapid descent of the glycemia is obtained on using them and since the course of coma treatment is improved in patients in which it was used.

At the same time, we must not disdain either in interpreting this action the insulin reinforcement of the Na cation, which acts as a sensitivity factor to insulin.

On the other hand, the observed fact of the low levels of glycemia without appearance of hypoglycemic coma might be attributed to the fact that the sodium β-glycerophosphate causes a decrease in the phosphatic activity of blood serum, and as is known, in insulin comas the phosphatases increase their level in the blood.

Conclusions

As a result of this first report we present the following:

1. Sodium β-glycerophosphate, sc. injected in fasting rabbits, behaves in a hypoglemic way.

2. It has an insulin reinforcement action which we attribute to the fact that sodium β-glycerophosphate, because it is a carrier and transmitter of phosphoric acid function, would act as activator of glycolysis.

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Instituto de Farmacología Experimental.—Sección de Madrid

ACCION DEL B-GLICEROFOSFATO SODICO SOBRE LA GLUCEMIA Y COMO MODIFICADOR DE LA ACCION INSULINICA

POR LOS DOCTORES

J. M. TORRES-ACERO FERNANDEZ y F. ANDOLZ AGUILAR

Los nuevos cauces que en la farmacología del B-glicerofosfato sódico ha abierto el profesor Velázquez y su escuela, al dar a conocer sus acciones espasmolíticas de la fibra uterina (1) (2) (3) y estimulante del centro respiratorio (4), han sido los motivos que nos han inducido a estudiar el comportamiento de este fármaco frente a la glucemia, así como las modificaciones que imprime sobre la hipoglucemia insulínica.

El papel metabólico del B-glicerofosfato sódico, ya ampliamente discutido en los trabajos citados, queda en parte comprobado por este nuestro, y nos permite deducir algunas nuevas aplicaciones del mismo que pueden tener interés clínico, como son, junto a la acción de refuerzo insulínico, su posible empleo en la prevención de los accidentes nerviosos de la insulinoterapia. En este sentido ya ha sido recomendado con este mismo fin el ácido adenosintrifosfórico, con parecida acción metabólica y comprobado en clínica psiquiátrica por Brazenstein y Lévi (5).

Método

Se han utilizado conejos adultos, sin distinción de sexo, sometidos previamente a un ayuno de veinticuatro horas, en los que se ha obtenido la sangre por sección de la vena marginal de la oreja. Las extracciones se han realizado de treinta en treinta minutos, después de la administración del B-glicerofosfato sódico o de la insulina por vía subcutánea.

La glucosa sanguínea se ha dosificado por el micrométodo de Folin-Malmros, por la ventaja que supone utilizar solamente 0,1 centímetros cúbicos de sangre, cuando a cada animal ha habido que hacerle en un mismo día casi veinte extracciones.

El método de Folin-Malmros es como sigue:

Se añaden 0,1 c. c. de sangre completa, tomada con buena pipeta de 0,1 c. c. de capacidad total, a 10 c. c. de ácido túngstico diluído, lavando la pipeta en el mismo por dos o tres veces; se mezcla y después de quince minutos de reposo se separa el líquido transparente por filtración.

En un tubo de Folin-Wu de 25 c. c. se depositan 4 c. c. del líquido transparente y se añaden 2 c. c. de la solución de ferricianuro potásico y 1 c. c. de la solución alcalina de cianuro. Se lleva el tubo a un baño de agua hirviente durante ocho minutos y se enfría seguidamente en un baño de agua fría. Se le añaden 5 c. c. de la mezcla fosfoférricagoma Ghatti y se deja en reposo durante dos minutos, completando después con agua destilada hasta 25 c. c.; se mezcla y después de diez minutos se hace lectura fotocolorimétrica, utilizando un filtro verde y disponiendo como solución de concentración cero, ajustada al 100 por 100 de transmisión un tubo de Folin-Wu con 4 c. c. de agua destilada, con el que se manipuló en igual forma que con los tubos problema.

Se construye la curva de trabajo preparando muestras de glucosa de distintas concentraciones, que son tratadas en la forma antes indicada para determinar el porcentaje de transmisión correspondiente a cada concentración conocida.

La preparación de los reactivos es como sigue:

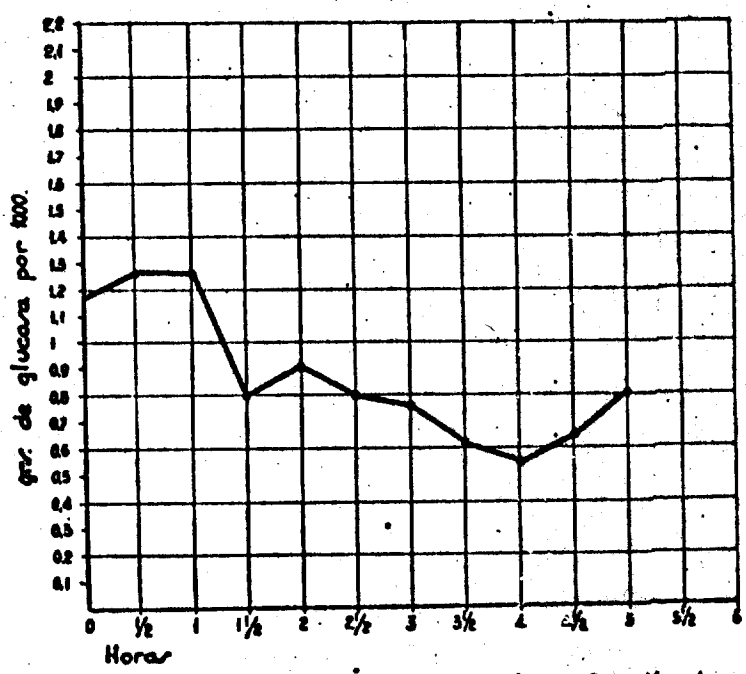
Acido túngstico diluído.—En un matraz aforado de 250 c. c. que contenga unos 200 c. c. de agua se añaden 5 c. c. de tungstato sódico al 10 por 100; después de mezclar se agregan lentamente 10 centímetros cúbicos de ácido sulfúrico 2/3 normal, agitando de nuevo y completando con agua hasta 250 c. c. Esta solución debe renovarse con frecuencia.

Solución acuosa de ferricianuro potásico al 0,4 por 100, conservada en frasco color topacio y renovada con frecuencia.

Solución alcalina de cianuro de sodio.—A 160 c. c. de solución de carbonato sódico anhidro al 10 por 100 se añaden 300 centímetros cúbicos de una solución recién preparada de cianuro sódico al 1 por 100, completando con agua destilada hasta 1.000 cen-

tímetros cúbicos. Es preciso extremar el cuidado en el manejo de esta solución tan tóxica.

Solución fosfoférricagoma Ghatti.—Se sumerge en un litro de agua destilada una bolsita cerrada de tela poco tupida que conten-

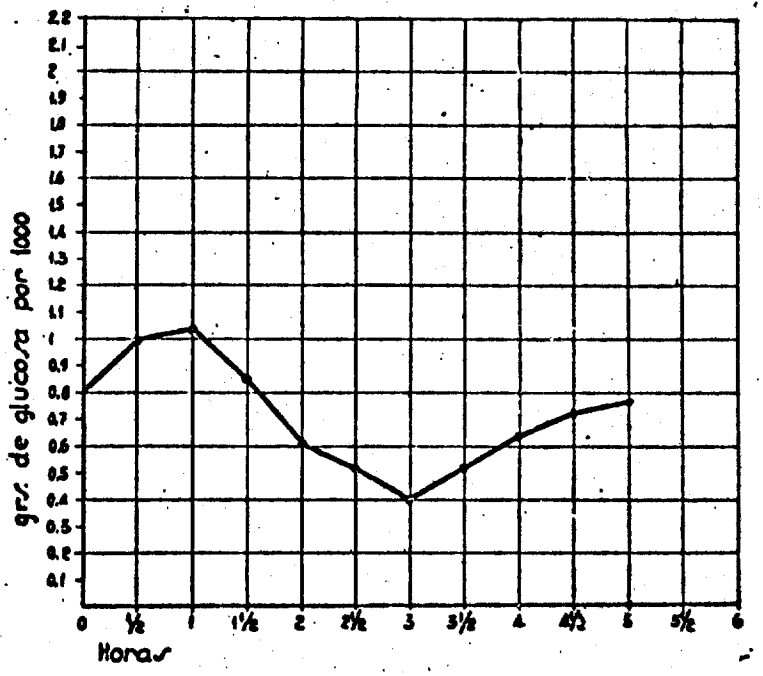


GRÁFICA NÚM. 1.—Conejo núm. 4, de 2,500 kilogramos de peso. Inyección subcutánea de 0,80 gramos de B-glicerofosfato sódico (0,057 gramos de P. orgánico) por kilo de peso. Valores de la glucemia: En ayunas, 1,18 gramos por 1.000; a los 30 minutos de inyectado el glicerofosfato, 1,27; a los 60 minutos, 1,27, 1,17; a los 90 minutos, 0,80; a los 120 minutos, 0,81; a los 180 minutos, 0,60; a los 150 minutos, 0,70; a los 210 minutos, 0,65; a los 240 minutos, 0,55; a los 270 minutos, 0,64; a los 300 minutos, 0,82.

ga 20 gramos de goma Ghatti, y se mantiene cerca de la superficie; a las dieciocho horas se filtra por una felpa, recogiendo todo el líquido filtrado, al que se le añaden cinco gramos de sulfato férrico disuelto en 75 c. c. de ácido fosfórico al 85 por 100 y 100 c. c. de agua destilada. Se añaden 20 c. c. de permanganato potásico al 1 por 100 y se mantiene a 37° durante cuarenta y ocho horas, quedando lista para su uso.

RESULTADOS

Efectos del B-glicerofosfato sódico sobre la glucemia.—Sobre la glucemia actúa el B-glicerofosfato sódico produciendo un claro des-



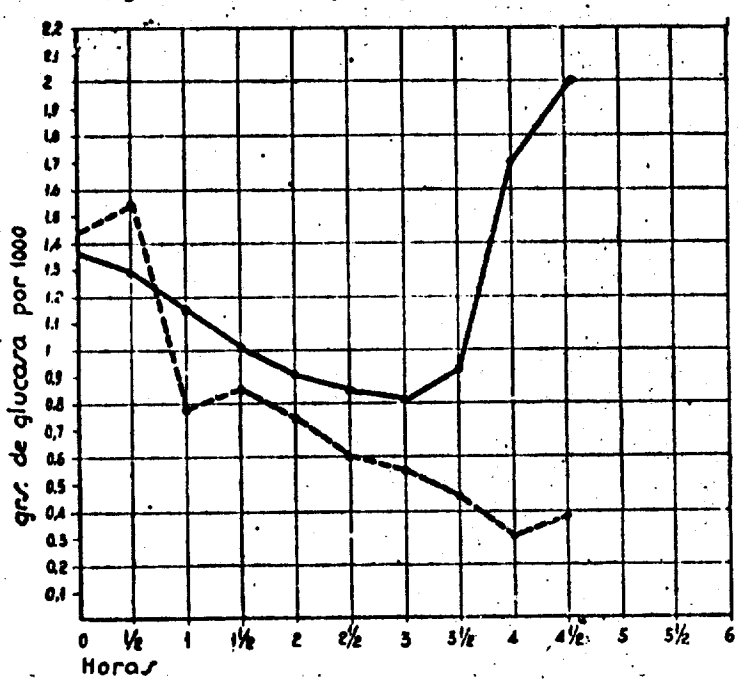
GRÁFICA NÚM. 2.—Conejo núm. 6, de 1,800 kilogramos de peso. Inyección subcutánea de 0,50 gramos de B-glicerofosfato sódico (0,057 gramos de P orgánico) por kilo de peso. Valores de la glucemia: En ayunas, 0,80 gramos por 1.000; a los 30 minutos de inyectado el glicerofosfato, 1,00; a los 60 minutos, 1,03; a los 90 minutos, 0,85; a los 120 minutos, 0,60; a los 150 minutos, 0,52; a los 180 minutos, 0,40; a los 210 minutos, 0,51; a los 240 minutos, 0,63; a los 270 minutos, 0,71; a los 300 minutos, 0,78.

ceso, previo un ligero incremento de la misma. El efecto se mantiene durante tres a cuatro horas, para recuperarse el nivel de la glucemia al cabo de este tiempo hasta su valor inicial (glucemia en ayunas).

Los valores más bajos de glucemia alcanzados han sido del orden

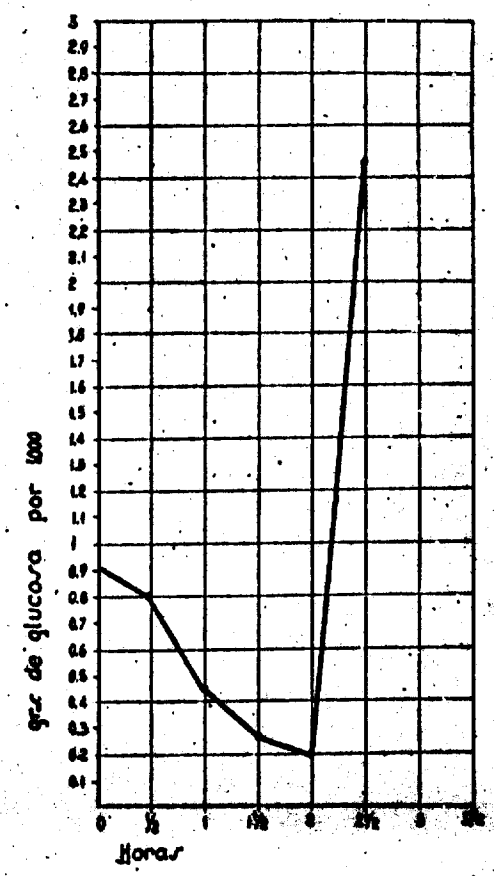
de 0,50 a 0,40 gramos por 1.000, sin presentarse en los conejos en ningún caso cuadro convulsivo.

En las gráficas números 1 y 2 representamos las curvas de glu-

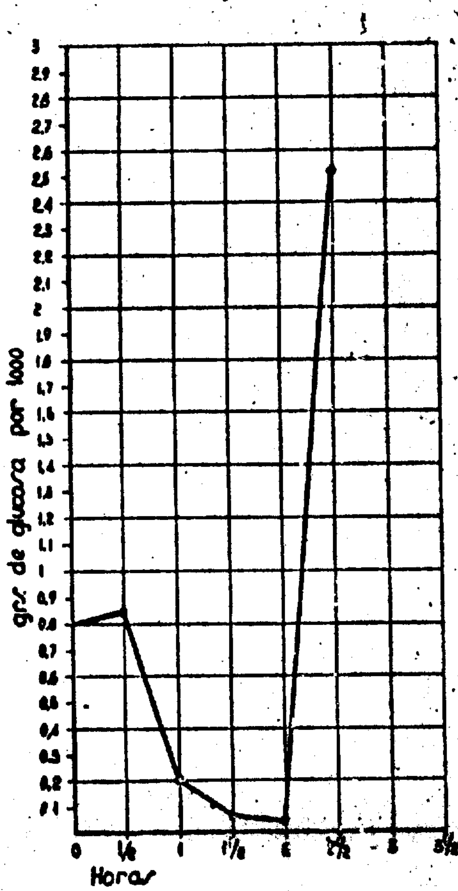


GRÁFICA NÚM. 3.—Conejo núm. 7, de 2,350 kilogramos de peso. Inyección subcutánea de 2 unidades de insulina por kilogramo de peso. Valores de la glucemia: En ayunas, 1,35 gramos por 1.000; a los 30 minutos de inyectada la insulina, 1,30; a los 60 minutos, 1,15; a los 90 minutos, 1,00; a los 120 minutos, 0,90; a los 150 minutos, 0,85; a los 180 minutos, 0,81; a los 210 minutos, 0,92; a los 240 minutos, 1,70; a los 270 minutos, 2,01. (Curva en negro).—El mismo conejo. Inyección subcutánea de 2 unidades de insulina por kilogramo de peso, más 0,50 gramos de B-glicerofosfato sódico por kilogramo de peso. Valores de la glucemia: en ayunas, 1,43 por 1.000; a los 30 minutos de inyectado el glicerofosfato e insulina, 1,55; a los 60 minutos, 0,77; a los 90 minutos, 0,86; a los 120 minutos, 0,75; a los 150 minutos, 0,60; a los 180 minutos, 0,55; a los 210 minutos, 0,46; a los 240 minutos, 0,36; a los 270 minutos, 0,88. (Curva en rojo.)

cemia obtenidas en los conejos 4 y 5, a los cuales les fué inyectado el B-glicerofosfato sódico por vía subcutánea, a razón de 0,50 gramos por kilogramo de peso, equivalente a 0,057 gramos de P orgánico.

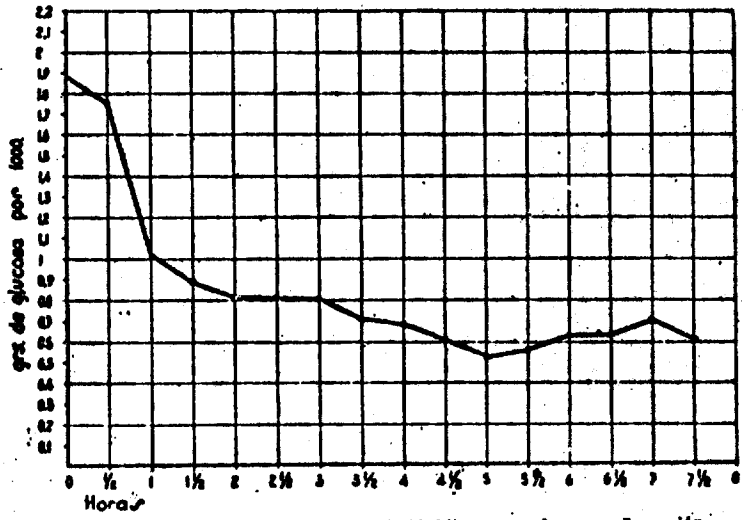


GRÁFICA NÚM. 4.—Conejo núm. 9, de 2,150 kilogramos de peso. Inyección subcutánea de 2 unidades de insulina más 0,75 gramos de B-glicerofosfato sódico por kilo de peso. A los 150 minutos se le inyectó 0,50 gramos de glucosa por kilo. Valores de la glucemia: En ayunas, 0,91 gramos por 1.000; a los 30 minutos de inyectado con glicerofosfato e insulina, 0,80; a los 60 minutos, 0,45; a los 90 minutos, 0,25; a los 120 minutos, 0,19; a los 180 minutos, 2,48.



GRÁFICA NÚM. 5.—Conejo núm. 10, de 1,750 kilogramos de peso. Inyección subcutánea de 2 unidades de insulina más 0,75 gramos de B-glicerofosfato sódico por kilogramo de peso. A los 150 minutos se le inyectó 0,50 gramos de glucosa por kilo. Valores de la glucemia: En ayunas, 0,80 gramos por 1.000; a los 30 minutos de inyectado con glicerofosfato e insulina, 0,84; a los 60 minutos, 0,21; a los 90 minutos, 0,06; a los 120 minutos, 0,05; a los 150 minutos, 2,50.

Efectos de la asociación B-glicerofosfato sódico-insulina sobre la glucemia.—Al emplear la insulina con el B-glicerofosfato sódico obtenemos un descenso muy marcado de la glucemia, llegando a niveles muy bajos de ésta. Comparando estos resultados con los obtenidos con insulina sola, claramente se observa que el descenso



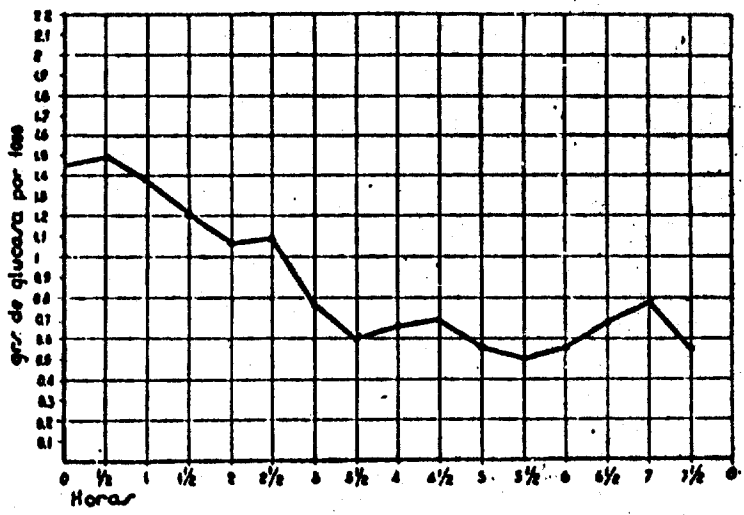
GRÁFICA NÚM. 6.—Conejo núm. 11, de 2,250 kilogramos de peso. Inyección subcutánea de 2 unidades de insulina, más 0,50 gramos de B-glicerofosfato sódico por kilo de peso. Valores de la glucemia: En ayunas: 1,87 gramos por 1.000; a los 30 minutos de inyectado con glicerofosfato e insulina, 1,75; a los 60 minutos, 1,01; a los 90 minutos, 0,88; a los 120 minutos, 0,82; a los 150 minutos, 0,81; a los 180 minutos, 0,80; a los 210 minutos, 0,71; a los 240 minutos, 0,69; a los 270 minutos, 0,60; a los 300 minutos, 0,62; a los 330 minutos, 0,63; a los 360 minutos, 0,63; a los 390 minutos, 0,68; a los 420 minutos, 0,68; a los 450 minutos, 0,60.

mayor y de más duración corresponde a la administración del B-glicerofosfato sódico con la insulina.

En la gráfica número 3 representamos las curvas de glucemia obtenidas en un mismo conejo, una por la administración de dos unidades de insulina por kilogramo de peso (curva en negro), y otra por la administración de insulina en la misma dosis, y B-glicerofosfato sódico, 0,50 gramos por kilogramo de peso (curva en rojo).

Este experiencia, en su segunda parte, fué realizada ya cuando el conejo se encontraba totalmente recuperado.

En las gráficas números 4 y 5 recogemos las curvas de glucemia obtenidas con los conejos números 9 y 10, en los cuales se inyectó una dosis algo mayor de B-glicerofosfato sódico (0,75 gramos por kilo de peso). En ellas podemos claramente apreciar los niveles tan bajos de glucemia alcanzados al administrar la insulina y el B-glicerofosfato. En estos dos casos fué necesario utilizar glucosa para que



GRÁFICA NÚM. 7.—Conejo núm. 14, de 2,000 kilogramos de peso. Inyección subcutánea de 2 unidades de insulina, más 1,25 miligramos de adenosiatrifosfato sódico (0,045 gramos de P. orgánico) por kilogramos de peso. Valores de la glucemia: En ayunas, 1,44 por 1.000; a los 30 minutos de inyectado con ATP e insulina, 1,48; a los 60 minutos, 1,37; a los 90 minutos, 1,20; a los 120 minutos, 1,00; a los 150 minutos, 1,07; a los 180 minutos, 0,77; a los 210 minutos, 0,50; a los 240 minutos, 0,38; a los 270 minutos, 0,68; a los 300 minutos, 0,55; a los 330 minutos, 0,80; a los 360 minutos, 0,56; a los 390 minutos, 0,65; a los 420 minutos, 0,77; a los 450 minutos, 0,55.

se recuperaran del coma hipoglucémico que se les produjo, realizándose esta recuperación con gran facilidad por la inyección subcutánea de 0,50 gramos de glucosa por kilogramo de peso.

La gráfica número 6 la presentamos para demostrar que este efecto hipoglucemiante es de duración larga. En esta experiencia la glucemia se mantenía a niveles bajos después de ocho horas de haber sido administrado el B-glicerofosfato sódico.

También señalaremos que al utilizar el B-glicerofosfato sódico con insulina el efecto inicial hiperglucemiante ligero que indicamos por el empleo del B-glicerofosfato solo o no aparece o si lo hace es casi de forma imperceptible (gráficas números 3, 4, 5 y 6).

Como ya indicamos anteriormente, los resultados obtenidos con el B-glicerofosfato sódico son muy semejantes con los que se presentan utilizando el ácido adenosintrifosfórico; la gráfica número 7 representa la curva glucémica por la administración de adesintrifosfato sódico a dosis de 1,25 miligramos por kilogramo de peso (equivalente a 0,045 gramos de P orgánico) más insulina a dosis de dos unidades por kilogramo de peso.

Discusión

Los resultados obtenidos por nosotros sobre la glucemia por la administración del B-glicerofosfato sódico hay que atribuirlos, sin duda alguna, al importante papel que esta sustancia posee en el metabolismo de los hidratos de carbono; recordemos el interés que el ácido glicerofosfórico tiene en el ciclo de Meyerhof.

El que al ácido adenosintrifosfórico se le considere en la actualidad, según los últimos trabajos de Cori y colaboradores (6), necesario junto a la enzima hexoquinasa en el mecanismo de acción de la insulina, nos lleva a pensar si el B-glicerofosfato sódico actuaría igualmente, facilitando la transformación de la glucosa en glucosa-6-fosfato (éster de Robinson).

De esta transformación sería responsable el B-glicerofosfato sódico, pues como éster fosfórico actuaría de transmisor o mediador de ácido fosfórico, siendo, pues, un activador de los substratos de glicólisis. Consideramos, por lo tanto, como indispensable en esta acción del B-glicerofosfato a la fracción fosfatada. Corroboran esta suposición nuestra los trabajos de Franks y colaboradores (7) (8), que recomiendan el uso del fosfato sódico en el coma diabético, pues han demostrado la existencia de un déficit de fosfatos en este cuadro, logrando un descenso rápido de la glucemia al emplearlos y siendo el curso del tratamiento del coma mejor en los enfermos en que fué utilizado.

Al mismo tiempo tampoco hemos de despreciar al interpretar

esta acción el refuerzo insulínico al catión *Na*, el cual actúa como factor de sensibilidad a la insulina.

Por otro lado, el hecho observado de los bajos niveles de glucemia sin aparición de coma hipoglucémico podría atribuirse a que el B-glicerofosfato sódico hace disminuir la actividad fosfatásica del suero sanguíneo, y como es sabido, en los comas insulínicos las fosfatasas aumentan su nivel en sangre.

Conclusiones

Como resultado de esta primera comunicación presentamos las siguientes:

1.ª El B-glicerofosfato sódico, inyectado subcutáneamente en conejos en ayunas, se comporta como hipoglucemiante.

2.ª Tiene una acción de refuerzo insulínico que nosotros atribuímos a que el B-glicerofosfato sódico, por ser portador y transmisor de la función ácido fosfórico, actuaría como activador de la glicólisis.

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THE PHARMACOLOGY OF SODIUM GLYCEROPHOSPHATE

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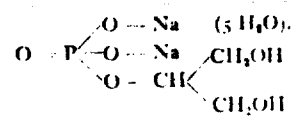
(Manuscript received August 9, 1948)

I

In every Institute of Pharmacology a series of works taking up more or less disparate topics is always in progress, and of these problems, studied together with a team of collaborators, there are some that are nearer completion, and a more exact vision can be given of these. Among the three matters which we consider most interesting in our work with BAYO, with GARCIA DE JALON, with ELIO and with ANDOLZ, are the pharmacology of novocaine or procaine in its pituitary sensitizing action and in the study of its various fractions (benzoic p-amino acid and ethanol amino diethyl); the study of a histaminolytic bacteria which we isolated from the atmosphere, freeing a histaminasic enzyme in its cultures; and the pharmacology of the polyalcohols, glycerine and sodium glycerophosphate. This problem is most near completion and we have already obtained excellent results in the clinic.

II

The glycerophosphates were known by the chemists of the past century, but it is only since the research of WILLSTATER and LUDECKE (1904) that we know their structure exactly. Of all the possibilities of combination of glycerine and phosphoric acid, there are two that interest us most at this time: sodium α-glycerophosphate and sodium β-glycerophosphate. The latter, with two secondary alcohol groups, has been shown to be of more interest pharmacologically.



Its neutral sodium salt is more stable and water-soluble. The pharmacology and therapeutics of the glycerophosphoric group, both as concerns its sodium or calcium modality, the most common

forms of its usage in therapeutics since the middle of the last century, has been reduced to its utilization in nutritional ailments, administered either through the digestive tract, most frequently calcium, or by means of injections, as is the case with sodium. In the case of chronic artheropathies of stiffening, LUCHERINI and Y. ROBERT (1943), maintain that the method of Rinaldi, combined with the intravenous administration of sodium glycerophosphate, provide a united alkalinotherapy. It is also used in the case of autospongiosis, together with the method of MAGGIOROTI. BOSSE recommends its use in paroxistic hemoglobinuria. As concerns this, it can be said that application of glycerophosphate has been reduced since GARCIA DE JALON and PEREZ CARNICERO (copyrighted) and I found another very interesting modification of its pharmacology.

III

Sodium beta-glycerophosphate as a uterospasmolytic. In continuation of our studies concerning the spasmolysis of glycerine and polyalcohols in general (1942-43), we initiated a study of spasmolytic action of glycerophosphates on smooth fiber. Insofar as glycerine acts in general as a spasmolytic of all smooth muscle, sodium glycerophosphate leads to the cessation of the spasms only in the uterine musculature, from which we infer that it has a utero-relaxing action.

When the uterus of a guinea pig, rat or rabbit reaches a stage of spasms due to histamine, acetylcoline, pylocarpine, pituitrine or baryum chloride, administering sodium beta-glycerophosphate in a bath in a concentration of 1/150 to 1/500, is sufficient to produce complete relaxation, with disappearance of the spasms, and only normal contractions, much reduced in comparison with those produced by the experiment, remaining. If the concentration of glycerophosphate is lesser (1/500 to 1/1500), the relaxation is induced, and the contractions closely resemble normal ones. With magnesium glycerophosphate, the spasmolytic action also succeeds, but the normal contractions also disappear.

It is interesting that other glycerophosphates, such as that of zinc, manganese, calcium, iron, etc., produce contractions and spasms instead of relaxation; from this it can be deduced that in these cases the action of the cation (Zn; Mn; Ca; Fe; etc.) predominates over that of the glycerophosphoric anion. The spasmolytic action of sodium glycerophosphate was also demonstrated in the human ovarian trunk by RONCALES (1947).

IV

Sodium beta-glycerophosphate as a respiratory analeptic. We were also able to observe in these studies that, when an animal's respiratory centers are intoxicated (anesthesia with chloral-morphine hydrate), intravenous injections of a 50% solution of sodium glycerophosphate (1 to 5 cc) has a considerable stimulatory effect on respiration: greater number of breaths of greater magnitude. This effect, which is notable when the intravenous route is used,

is nonetheless more sudden and more potent when the glycerophosphate is administered by the suboccipital route. In this case, it is an effect similar to that obtained lobelin administered intravenously or a potassium salt (potassium chloride, 1 to 2 centigrams) suboccipitally.

Thus, we believe that this sodium glycerophosphate can be included among respiratory analeptic medicines, since in all the cases of toxemia in respiratory centers, the results obtained by administration through the simple intravenous route can be excellent.

V.

These two pharmacological facts, uterospasmolysis and respiratory analeptics, call for the use of this drug in human clinical medicine. The first works of the obstetric school of BOTELLA LLUSIA, continued over these recent years (1944-48), demonstrate the good obstetrical results that can be obtained with this medication:
a) in the case of premature or dangerous miscarriages. If there is no dilatation of the neck, and if the egg is not dead, pregnancy continues; in the opposite case, the contractions are regularized, the spasms (spasmodic contractions) cease, and the expulsion of the egg or the fetus occurs easily and with almost no pain.
b) in the case of premature births, with the same reasons and basis as in the former indication.
c) in the case of dynamic or hypertonic dystocias, the intravenous injection of sodium beta-glycerophosphate causes the uterine contraction to disappear, the contractions normalize, and the delivery follows its normal course.
d) In the case of placenta retentions due to annular uterine spasms (placenta clampings), the injection of a sufficient amount of glycerophosphate causes the anular uterine spasm to disappear and the placenta exits without difficulty.
e) in the case of puerperal afterpain or postpartum pains, which cease easily upon the first injection of the preparation.

There is no toxicity, and only in the case of thiamin deficiencies the vitamin can be used in association with the glycerophosphate to reinforce its effects.

The research of other schools, such as that of GARCIA MARRUZ in Cuba, the Maternidad Alfredo da Costa of Lisbon, etc., are of the same opinion, and with this simple medicine very favorable results can be obtained in clinical obstetrics. Intravenous injections of sodium beta-glycerophosphate at 50% in distilled water, in a quantity of 5 to 20 cc, with repetition as considered necessary, are the effective posology of these indications.

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Velasquez-'48
Bib No. 391

CÁTEDRA DE FARMACOLOGÍA EXPERIMENTAL
DE LA FACULTAD DE MEDICINA. MADRID

LA FARMACOLOGÍA DEL GLICEROFOSFATO SÓDICO

B. LORENZO VELÁZQUEZ (*)

(*Manuscrito recibido el* 9-8-1948).

I

En cada Instituto de Farmacología hay siempre en marcha una serie de trabajos que abordan puntos más o menos dispares, y de estos problemas estudiados junto con el equipo de colaboradores, hay unos más acabados, y sobre ellos puede darse una visión de conjunto más exacta. De los tres puntos que con más interés tenemos en trabajo con BAYO, con GARCÍA DE JALÓN, con ELIO, con ANDOLZ, figuran la farmacología de la Novocaina o procaina en su acción pituitrino-sensibilizante y en el estudio de sus diversas fracciones (ácido p. amino benzóico y dietil amino etanólica); el estudio de una bacteria histaminolítica aislada por nosotros de la atmósfera, liberando en sus cultivos una enzima histaminásica; y la farmacología de los polialcoholes, glicerina y glicerofosfato sódico. Este problema está más acabado y ya ha dado en la clínica excelentes resultados.

II

Los glicerofosfatos fueron conocidos de los químicos del siglo pasado, pero justamente hasta los trabajos de WILLSTÄTER y LÜDECKE (1904) no se conoce exactamente su extructura. De todas las posibilidades de combinación de la glicerina con el ácido fosfórico, dos son las que más

(*) Hommage au Prof. E. ROTHLIN, à l'occasion de son 60e anniversaire.

nos interesan en este momento : el α glicerofosfato sódico y el β glicerofosfato sódico. Este último, con dos grupos alcoholicos secundarios, es el que farmacológicamente se nos ha mostrado de mayor interés. Su sal sódica neutra es muy estable y soluble en agua. La farmacología y terapéutica del grupo glicerofosfórico, ya sea en su modalidad sódica o cálcica, formas las más comunes de su empleo en la terapéutica desde mediados de la pasada centuria hasta nuestros dias, quedaba reducida a su utilización en enfermedades de la nutrición, ya por via gastrica, el cálcico con más frecuencia, o bien en inyecciones, el sódico sobre todo. En artropatias crónicas anquilosantes constituyó el Método de Rinaldi, y en relación a ello, recientemente LUCHERINI é Y. ROBERT, sostienen (1943) que al dar intravenoso el glicerofosfato sódico se haría una alcalinoterapia intraarticular. Se usó también en otoespongiosis, y así constituyó el método de MAGGIOROTI. BOSSE lo recomendó en la hemoglobinuria paroxística. A esto puede decirse que se reducen las aplicaciones del glicerofosfato hasta que con GARCÍA DE JALÓN y con PEREZ CARNICERO (Amparo), encontramos otra modalidad muy interesante de su farmacología.

III

El beta glicerofosfato sódico como úteroespasmolítico. Como continuación de nuestros estudios sobre la espasmolisis de la glicerina y de los polialcoholes en general (1942-43), iniciamos el estudio de la acción espasmolítica sobre fibra lisa de los glicerofosfatos. En tanto la glicerina se comporta en general como espasmolítica de toda la musculatura lisa, el glicerofosfato sódico produce la cesación de los espasmos solamente sobre la musculatura uterina, de donde se infiere que tiene una acción específica úterorelajadora.

Cuando un útero de cobaya, de rata o de coneja, se lleva a un estado de espasmo por la histamina, acetilcolina, pilocarpina, pituitrina, o cloruro de bario, basta agregar beta glicerofosfato sódico en concentración del baño de 1/150 a 1/500, para que se advierta una relajación completa, con desaparición del espasmo, quedando las contracciones normales muy reducidas en comparación a las anteriores a la experiencia. Si la concentración del glicerofosfato es menor (1/500 a 1/1500), la relajación se obtiene y las contracciones se asemejan mucho a las normales. Con el glicerofosfato magnésico la acción espasmolítica se logra, pero las contracciones normales desaparecen también.

Es interesante que otros glicerofosfatos, como el de zinc, el de manganeso, el de calcio, el de hierro, etc., producen contracciones y espasmos en lugar de relajación; de ello puede deducirse que aquí domina la acción del catión (Zn; Mn; Ca; Fe; etc.) sobre la del anión glicerofosfórico. La acción espasmolítica del glicerofosfato sódico fué demostrada también *para la trompa ovárica humana* por RONCALÉS (1947).

IV

El beta glicerofosfato sódico como analéptico respiratorio. También pudimos observar en estos estudios, que cuando un animal se encuentra con sus centros respiratorios intoxicados (anestesia con hidrato de cloral-morfina), las inyecciones intravenosas de una disolución al 50% del glicerofosfato sódico (1 a 5 c.c.) determina una considerable acción estimulante sobre la respiración: mayor número de respiraciones y mayor amplitud de las mismas. Esta acción que ya se acusa notablemente cuando se utiliza la vía intravenosa, es todavía más brusca y más potente cuando al glicerofosfato se administra por la vía suboccipital. Es una acción en este caso parecida a la que se obtiene con la lobelina intravenosa o con una sal de potasio (cloruro potásico, 1 a 2 centigramos) suboccipital.

Así, pues, creemos que este glicerofosfato sódico puede ser incluido entre los medicamentos analepticos respiratorios, pues en todo caso de toxemia sobre centros respiratorios, los resultados por la sencilla vía intravenosa pueden ser excelentes.

V

Estos dos hechos farmacológicos, la úteroespasmolisis y la analéptica respiratoria, permiten el empleo de este fármaco en la clínica humana. Ya los trabajos primeros de la escuela obstétrica de BOTELLA LLUSIÁ, proseguidos después a lo largo de estos años (1944-1948), demostraron los buenos resultados en obstetricia que podían obtenerse con este medicamento : *a*) en abortos incipientes o amenazantes. Si la dilatación del cuello no está hecha, y si el huevo no está muerto, el parto continúa; en caso contrario, las contracciones se regularizan, los espasmos (contracciones espasmódicas) ceden, y la expulsión del huevo o del feto tiene lugar con facilidad y sin apenas dolores. *b*) En partos prematuros con las mismas razones y fundamento que en la anterior indicación. *c*) En

distocias dinámicas o hipertónicas la inyección intravenosa del beta glícerofosfato sódico hace desaparecer la contractura uterina, las contracciones se normalizan, y el parto sigue su curso normal. *d*) En las retenciones placentarias por espasmo anular uterino (engatillamientos placentarios) la inyección de la suficiente cantidad de glícerofosfato hace desaparecer el espasmo anular uterino y la placenta sale con facilidad. *e*) En los entuertos puerperales o dolores postpartum, que ceden con facilidad a la primera inyección del preparado.

La toxicidad es nula, y sólo en los casos de insuficiencias tiamínicas, debe asociarse esta vitamina para reforzar sus acciones.

Los trabajos de otras escuelas, como la de GARCIA MARRUZ en Cuba, de la *Maternidad Alfredo da Costa*, de Lisboa, etc., abogan en el mismo sentido, y hace que de este sencillo fármaco se puedan obtener muy buenos resultados en la clínica obstétrica. Las inyecciones intravenosas del beta glícerofosfato sódico al 50 % en agua destilada, y en cantidad de 5 a 20 c.c., con repetición de las veces que se crea conveniente, constituyen la posología eficaz para estas indicaciones.

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WORTON- 50
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Comparative Evaluation of Calcium Glycerophosphate As a Dietary Calcium Supplement*

ALBERT G. WORTON, THEODORE E. BYERS and JAMES M. GILLINGHAM

IN STUDYING the extent of calcification of bone in mice produced by a basic diet supplemented with various calcium compounds in equivalent amounts of calcium, calcium glycerophosphate was found to produce the highest degree of bone calcification. Other calcium compounds in decreasing order of efficiency were tricalcium phosphate, calcium lactate, dicalcium phosphate and calcium carbonate. The *in vitro* behavior of various calcium compounds through the gastrointestinal *p*H range was observed.

Recent trends in calcium supplementation have raised the following questions: (1) Is dicalcium phosphate an inadequate calcium supplement? (2) Are phosphorus-free calcium compounds ideal as sources of calcium? (3) Is phosphorus important for calcium retention? (4) Which of all the well-known calcium compounds is most efficient in bone development?

The increased nutritional requirements for calcium, particularly during pregnancy and lactation, have been recognized for many years and are now generally accepted.[1] Authorities maintain that an adequate calcium level by dietary means during this period is improbable.[2,3] The type of calcium and criteria for evaluating the efficiency of various calcium compounds appear to require further study.

DISCUSSION

Dicalcium phosphate in particular has been widely administered to pregnant and lactating women. Unfortunately, according to medical reports in recent years, the administration of dicalcium phosphate has been shown to contribute to neuromuscular irritability as manifested by leg cramps in pregnant women.[4] From these results and associated blood studies it has been generally concluded that insoluble phosphates have a depressive effect on absorption of calcium from the intestinal tract.

Other findings indicate that administration of dicalcium phosphate may not elevate the serum or the ionized calcium levels, and that it is inefficient as a calcium supplement.[5,6] It has been shown that ingested calcium frequently is rendered soluble by stomach acid but is then precipitated by the increasing alkalinity of the intestine before absorption can occur.[7] Some authors have reported that as little as 10 per cent of ingested calcium is absorbed.[8]

Because of poor results with dicalcium phosphate, there has been a tendency to use phosphorus-free calcium compounds. As a matter of fact, the lactate, gluconate and carbonate may be used to significantly raise the calcium blood level. Although neuromuscular irritability may be reduced with such compounds this does not guarantee skeletal metabolism of calcium. Indeed, the calcium may be on a one-way street to excretion. Authorities believe the level of the blood calcium to indicate only the height of the stream and not the direction of the flow.[8] It is also true that a calcium deficiency may exist despite a normal blood calcium, since calcium storage in the tissues may be depleted before hypocalcemia develops.[9] Therefore, blood calcium readings cannot be accepted as proof of normal levels of tissue calcium.

The futility of attempting to increase calcium retention with phosphorus-free calcium salts alone has been demonstrated under controlled experimental conditions.[9] A diet that permits normal deposition of calcium in the bone may be transformed to a rickets-producing diet by merely increasing dietary calcium and allowing dietary phosphorus to remain constant.[10] Interestingly enough, phosphorus-free calcium salts are used to hasten the development of experimental rickets.[11,12] The severity of the rickets produced under these conditions increases in ratio with the increasing disproportion of dietary calcium and phosphorus.

The primary purpose of calcium supplementation is to achieve calcification of tissue and this cannot be accomplished without simultaneous phosphatification.[10,13] The failure of absorbed calcium to be used in calcification is said to be due to a low blood phosphorus level and calcium absorption is most rapid when phosphorus absorption is highest.[14] Calcium fixation in the skeleton is thus dependent upon a simultaneous fixation of phosphorus.[15]

It is well to recall that the minimum daily

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requirement for phosphorus is approximately equal to that for calcium.[14, 17] Some antirachitic substances act by elevating blood phosphorus, thus increasing calcium deposition.[14] The two elements have been shown to be so closely interrelated that the utilization of one cannot be considered without that of the other.[18]

Because of the apparent inefficiency of some calcium compounds comparative evaluation of those commonly used obviously requires further study. Also, because of the hand in hand metabolism of calcium and phosphorus it appeared to be worth while to discover a calcium and phosphorus compound that is efficiently absorbed from the intestine and differs from dicalcium phosphate in that it does not cause neuromuscular irritability.

With this in mind, a number of calcium compounds were studied, including dicalcium phosphate, tricalcium phosphate (bone ash), calcium lactate, calcium gluconate, calcium carbonate and calcium glycerophosphate. The first five are the principal calcium supplements in use today. Calcium glycerophosphate until now had been employed mainly as a source of glycerophosphoric acid for use as a nerve tonic in debility and neurasthenia.[18] The efficiency of calcium glycerophosphate as a source of calcium over the many years of its use, thus far, has gone virtually unnoticed.

Canals and his associates fed radioactive compounds to animals and followed the route of assimilation. They found that calcium glycerophosphate gave the highest bone absorption figures and was the most efficient of the calcium compounds tested.[19]

Lecoq developed experimental rickets with a phosphorus-free diet containing 1 per cent calcium lactate to hasten the rachitogenic effect.[20] Phosphorus-containing calcium compounds were required to cure the rickets in the following minimum amounts:

	Min. Amt. Needed
Calcium Glycerophosphate	0.85 Gm.
Dicalcium Phosphate	1.55 Gm.
Tricalcium Phosphate (bone ash)	2.50 Gm.

They showed the inorganic calcium phosphates to be largely wasted in contrast to the high efficiency of the organic calcium glycerophosphate.[20]

The dosages of the various calcium compounds reflect their relative efficiencies. The U. S. P.[21] or N. F.[22] recommend the following dosages:

Calcium Glycerophosphate N. F. X	0.6 Gm. (9 grs.)
Dicalcium Phosphate U. S. P. XV	1.0 Gm. (15 grs.)
Tricalcium Phosphate N. F. X (Technical grade is bone ash)	1.0 Gm. (15 grs.)
Calcium Carbonate U. S. P. XV	1.0 Gm. (15 grs.)
Calcium Lactate N. F. X	5.0 Gm. (75 grs.)
Calcium Gluconate U. S. P. XV	5.0 Gm. (75 grs.)

Thus, with the greater absorptive efficiency of calcium glycerophosphate, a lesser dosage schedule than with other calcium compounds is possible.

The work of Canals and Lecoq and the official dosage recommendations for the several calcium compounds studied, indicate the superior efficiency of the glycerophosphate of calcium. The present study is designated to compare the efficiency of several commonly used calcium compounds with that of calcium glycerophosphate.

EXPERIMENTAL

In vitro experiments were performed to demonstrate the precipitation of various calcium compounds in the presence of phosphate at the *pH* levels prevalent in the gastrointestinal tract.

Concentrations of calcium glycerophosphate, dicalcium phosphate, calcium lactate, calcium gluconate were prepared in distilled water to contain 3 mg. of elemental calcium per milliliter. To the calcium lactate and calcium gluconate, phosphate ion was added to equal the 2.32 mg. per ml. of phosphorus concentrations of both the calcium glycerophosphate and the dicalcium phosphate. Portions of each of these solutions were adjusted through the *pH* range 2 to 9 with hydrochloric acid or sodium hydroxide. Figure 1 (see following page) shows calcium glycerophosphate to be soluble at all *pH* levels 2 through 9. Precipitation is observed at *pH* 3 with dicalcium phosphate, *pH* 4 with calcium lactate, and *pH* 5 with calcium gluconate.

Based on the work of others, the hydrogen ion concentration of the stomach, duodenum and ileum are *pH* 2, *pH* 6 and *pH* 8, respectively.[23, 24, 25, 26]

It is noteworthy that calcium glycerophosphate overcomes the chief objection to other phosphate-containing calcium supplements in that it is soluble throughout the gastrointestinal *pH* range.

The wide range of solubility of calcium glycerophosphate suggests a greater efficiency of calcification in animal tissue and indicated the need for actual evaluation in this respect in comparison with other calcium compounds.

Adequate gastric acid is essential for most calcium compounds to be absorbed. Since calcium compounds are usually antacid, it is important to select one that has a minimal acid depressing action. Goodman and Gilman stress this point: "An acid reaction in the intestine promotes calcium absorption by favoring solution of calcium salts, whereas an alkaline (antacid) reaction precipitates calcium and decreases absorption."

On a strict comparative basis, 500 mg. of the various calcium salts depress acidity as follows:

Calcium Carbonate	200.00 cc.
Tricalcium Phosphate	128.00 cc.
Dicalcium Phosphate	61.00 cc.
Calcium Lactate	55.00 cc.
Calcium Glycerophosphate	45.75 cc.

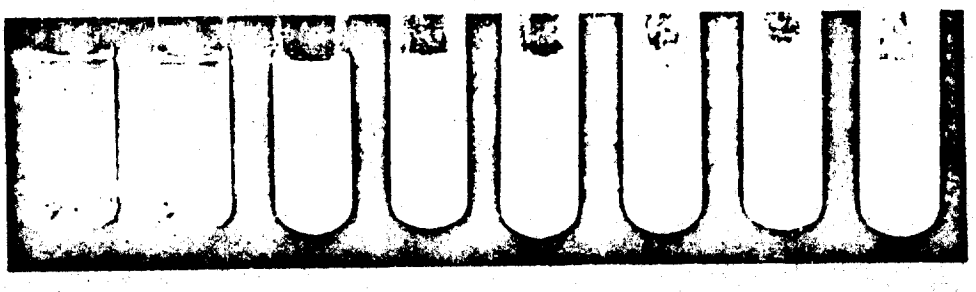
(Using 0.05 N. Hydrochloric Acid and Bromphenol Blue as indicator)

Having minimal antacid properties, the glycerophosphate is an ideal calcium supplement because

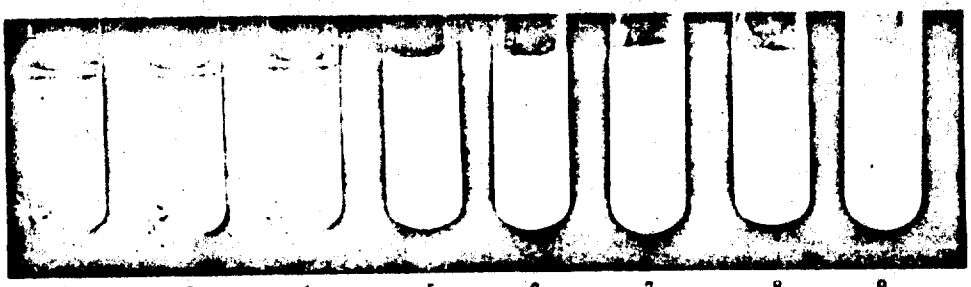
CALCIUM GLYCEROPHOSPHATE

DICALCIUM PHOSPHATE

CALCIUM LACTATE WITH PHOSPHATE



CALCIUM GLUCONATE WITH PHOSPHATE



pH 2 3 4 5 6 7 8 9

NOTE: All tubes contain equivalent amounts of calcium and phosphorus.

Fig. 1. Precipitation of several calcium compounds in the presence of phosphate at various pH levels.

of the frequent acid deficiencies of both obstetric and geriatric patients.

ANIMAL STUDY*

Newly weaned female mice were separated into six groups. A diet based on the U. S. P. XIV Rachitogenic Diet No. 1, but omitting calcium carbonate, was prepared. The diet consisted of the following:

Whole Wheat Ground	34%
Whole Yellow Maize	34%
Gelatin	16%
Ground Wheat Gluten	15%
Sodium Chloride U. S. P.	1%

The animals were fed this basic diet for two weeks. At the end of the two weeks, the diet was divided into six parts, one group continuing on the basic diet and different calcium compounds added to the other five parts as follows:

A—0.5% Calcium as Calcium Carbonate.
B—0.5% Calcium as Calcium Lactate.
C—0.5% Calcium as Dicalcium Phosphate.
D—0.5% Calcium as Tricalcium Phosphate (bone ash).
E—0.5% Calcium as Calcium Glycerophosphate.
F—Control.

The mice were fed ad libitum. Distilled water was furnished for drinking.

Samples of the leg bones (femurs and tibias) were cleaned and dried to constant weight at 110°C. The femurs and tibias were then tested for relative breaking strength by compressing each bone in a vertical position in a Strong Cobb tablet hardness tester. The dried bones were then assayed for calcium.[2] The percentage of bone calcium is shown in figure 2. The relative breaking strengths are shown in figure 3.

There were some striking differences in the appearance of the several groups of mice after a six week course of supplementation with various types of calcium. Those mice on the calcium glycerophosphate supplement were sleek in appearance and fully coated. The group receiving calcium carbonate exhibited extreme nervousness and excitability, marked loss of hair and scabbing, particularly on the face and tail (Fig. 4). Loss of hair around the face and back was also noted in a number of the mice receiving calcium lactate and, to a lesser degree, those receiving tricalcium phosphate. The control and dicalcium phosphate groups, though not exhibiting the sleek appearance of the calcium glycerophosphate mice, showed little or no tendency toward loss of hair or scabbing.

ANALYSIS OF RESULTS

The mice maintained on a basic diet supplemented with calcium glycerophosphate consist-

*The authors are indebted to Mr. Kenneth A. Teague, now with the Department of Physiology, Ohio State University, for invaluable aid with the animal work.

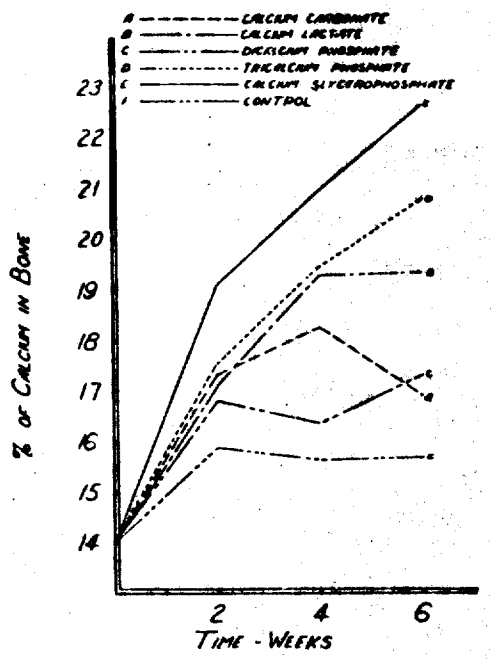


Fig. 2. Percentage of bone calcium obtained with various calcium supplements.

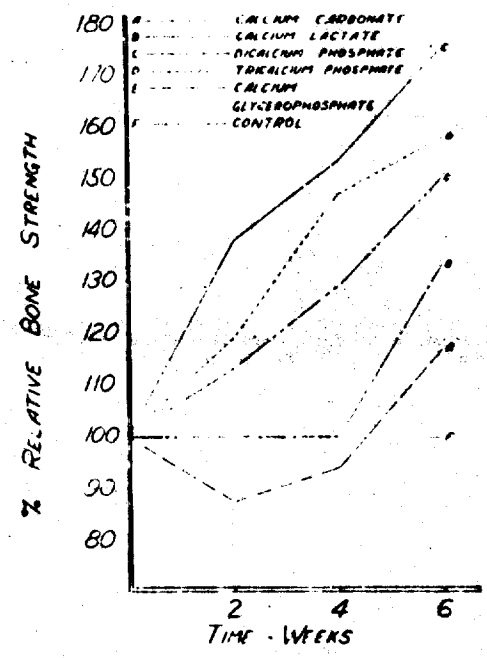


Fig. 3. Average increase of femur strength of mice on various calcium supplements using bone strength of control mice as 100 per cent.