raccoon hepatitis, if such it is, may bear to infectious hepatitis of man.

Summary. An infectious agent, which appears to be a virus (RJV) has been isolated from the liver of a wild raccoon which has led to a highly fatal type of disease characterized by conjunctivitis and an elevated serum bilirubin frequently accompanied by jaundice on inoculation of raccoons. Ferrets also appear to be susceptible to infections with this agent.

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Calcification. XII. Cation-Linked Inhibition by Fluoride and Cyanide Ions in β-Glycerophosphate Medium.* (20853)

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In a previous report it was shown that in the presence of magnesium ions in solutions employed for calcification *in vitro,* both cyanide and fluoride ions exert a marked inhibitory effect. In the absence of magnesium ions, however, fluoride actually enhances the degree of mineralization, while cyanide appears to have neither a blocking nor an enhancing effect(1). Magnesium, in the amounts used, either exerted no measurable influence on the degree of mineralization or only a mild inhibition when cyanide or fluoride ions were not present. Recently Marks and his associates commented on the cyanide inhibition, stating that magnesium inhibition in itself accounts for the findings of cyanide inhibition(2). For this reason our experiments were carefully repeated and the data presented in Table IV of reference 1 were confirmed. It was shown at that time that only in the presence of magnesium ions added to the calcifying fluid will cyanide block the mineralizing process *in vitro.* The present work is concerned with an extension of the studies of cyanide and fluoride inhibition to calcifying media containing β-glycerophosphate as the source of phosphorus. In the course of these experiments it was found that cations other than magnesium, such as strontium, barium, and manganese, also cause cyanide to act as an inhibitor.

Robison and his co-workers(3,4) were first to observe inhibition of *in vitro* calcification by fluoride and cyanide ions in inorganic phosphate solution.‡ No cyanide effect was observed with β-glycerophosphate, although a block by fluoride was still evident. A reinvestigation of the problem by Gutman, *et al.* (5-7), served to confirm the earlier work of Robison, with one exception: neither cyanide nor fluoride interfered with calcification in mineral solutions containing phosphorus as β-glycerophosphate.‡ This point of discrepancy seemed to warrant further inquiry, particularly in view of the significance attached to the apparently dissimilar behavior of the ions in inorganic and organic phosphate medium(5-7).

Methods. A modified USP rachitogenic diet replaced the diet previously used(8). The rations, on which the Wistar strain albino rats were placed at the age of 22-24 days, consisted of 76% degermed corn meal, 20% wheat gluten, 3% $CaCO_3$, and 1% NaCl. The rachitic animals were sacrificed 17 to 23 days later. Slices of bone cartilage were removed from their tibiae and suspended in the designated solutions in 25 ml Erlenmeyer

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‡ In addition to calcium, phosphate, sodium, potassium, and bicarbonate, magnesium was included in the solutions of both Robison and Gutman to simulate its presence in serum. The critical role of magnesium in the inhibitory process has only recently been recognized(1).

TABLE 1. Relative Rates of Calcification in β-Glycerophosphate and Inorganic Phosphate Media. (Ca^{++}) = 10 mg % = 2.5 mM.

Mg^{++} conc., mM/L	Incubation period, hr	Degree of calcification: Organic P conc. 3 mg %	4 mg %	5 mg %	6 mg %	10 mg %	Inorganic P conc., 5 mg %
0	5		2.0(4+)	2.5(4+)	2.8(4+)		2.6(4+)
1	5		1.3(3+)	1.6(3+)	1.9(4+)		1.7(3+)
1	6	1(+)		1.5(4+)		2.2(4+)	1.6(4+)
1	18			2.2(4+)		3.2(4+)	2.4(4+)
1	18		1.8(4+)	2.3(4+)	2.8(4+)		2.6(4+)

flasks from glass hooks affixed to the underside of paraffined cork stoppers. The upper level of the solution was kept within 4-5 mm of the bottom of the stopper. Incubations were conducted without shaking at 37°C and pH 7.3. Additional experimental details are given in Papers V(1) and IX(9) of this series. Each line of the accompanying tables represents results obtained from 2 to 4 rats. The source of β-glycerophosphate was the Mallinckrodt disodium salt, theoretical mol. wt. 315.15 based on a water content of 5.5 moles. Analysis of the compound revealed it to be free of inorganic P. Following digestion with a nitric-sulfuric acid mixture, the P assayed almost 6% in excess of theory (digestion blanks and inorganic P controls were run simultaneously). Stock solutions of β-glycerophosphate containing 1 mg organic P per ml were prepared by dissolving 0.960 g of the reagent in distilled water and diluting to 100 ml. The new mineral deposit formed *in vitro* was revealed by silver staining. The degree of calcification was graded as follows: no calcification, 0(0); maximum calcification, 4(++++); the length of the line or deposit is designated by the +'s and its thickness or width by the number preceding the parenthesis (10). Since the width of the deposit is often not uniform, we have adopted the procedure of assaying each quarter of the line (under 60 X magnification) and then taking the average. Agreement was generally obtained between the mean values for the 2 sides of a given slice, and also for the 2 or 3 slices from each tibia.

Results. In order to assess the effect of cyanide and fluoride ions on calcification in β-glycerophosphate medium relative to their action in inorganic phosphate, it was first necessary to determine what levels of organic and inorganic phosphate yield equivalent amounts of mineral deposit. No systematic effort to fix this relationship appears to have been made by the earlier workers. The reference system used here, as well as in our previous study(1), consisted of basal salt (70 mM NaCl, 5 mM KCl, and 22 mM $NaHCO_3$ per liter), 10 mg % (2.5 mM) Ca^{++}, and 5 mg % (1.61 mM) P as inorganic phosphate. The test solutions contained 3 to 10 mg % P as β-glycerophosphate but were otherwise identical in makeup. Incubations were conducted for 5 to 18 hours in the presence and absence of Mg^{++}. As contrasted with the mineral deposit acquired by the slices of cartilage in 5 mg % inorganic P, the degree of calcification in organic phosphate was found to be markedly decreased at 3 to 4 mg %, approximately equal at 5 to 6 mg %, and greater at higher concentrations (Table I). From the tabulated data it was ascertained that 5 mg % inorganic P is equivalent to a mean value of 5.5 mg % β-glycerophosphate P.

Having established that rachitic rat cartilage calcifies to about the same extent in solutions 10/0/5.5§ and 10/5/0,§ incubations were carried out in organic medium to determine the effect of 1 mM KCN. No cyanide inhibition was noted, even in the 5-hour period (Table II). *Inhibition was obtained* in the cyanide flasks, however, when the solution was supplemented with 1 mM Mg^{++}. At the concentrations of Mg^{++} and cyanide employed here, the block of calcification was usually complete in the first 5 hours, but

§ The symbol a/b/c is used to represent a mineral solution containing the basal components and: a, mg% Ca^{++}; b, mg% inorganic P; and c, mg% organic P (here, β-glycerophosphate).

TABLE II. Magnesium-Dependence of Cyanide Inhibition in β-Glycerophosphate Medium. (10/0/5.5)

Mg^{++} conc., mM/L	Cyanide conc., mM/L	Incubation period, hr	Degree of calcification Test tissue	Control*
0	1	5	2.5(4+)	2.3(4+)
0	1	18	2.8(4+)	2.8(4+)
1	1	5	1 (1+)	1.8(4+)
1	1	18	1.9(2+)†	2.3(4+)

* No cyanide present in calcifying solution.
† Subsurface deposit.

rarely so after 18 hours of incubation. Similar observations have been recorded for the system 10/5/0(1).

Calcification in organic as well as inorganic (1) medium containing Mg^{++} was blocked in the presence of 10^{-4} to 10^{-3} molar fluoride (Table III), and more decisively than with cyanide ions. In contrast to the results obtained with cyanide, 10^{-4} molar fluoride proved to be inhibitory in the first 5 hours in the absence of added Mg^{++}, although calcification did occur within 18 hours. Less inhibition was noted at the 10^{-3} molar fluoride level after the first 5 hours, while in 18 hours the degree of calcification actually exceeded the control sections, but not markedly. Identical results were obtained in solution 10/5/0 containing 10^{-3} molar fluoride and no Mg^{++}. The less pronounced increase in deposition over the fluoride-free control obtained here in both organic and inorganic media, as compared to our previous experiments(1), may be a result of the change in the rat diet. The mineral deposit formed in fluoride solution could be visualized prior to silver staining as an opaque white band present below the epiphyseal plate. Microscopic examination following staining revealed a typical honeycomb structure which was restricted to the metaphysis and undoubtedly represents *bona fide* calcification(1).

It was of further interest to determine whether the Mg^{++}-linked inhibition by fluoride and cyanide could be overcome at higher phosphate levels, as appeared likely from the literature, and particularly whether an equivalent response would be evoked in inorganic phosphate and β-glycerophosphate media. As the P concentrations were raised above 8 mg % inorganic P and 10 mg % organic P, however, difficulty was encountered in attempting to equate organic to inorganic phosphate. In both cases extensive and often limiting deposits were obtained in which the areas could not be easily differentiated. For this reason a calcifying solution was sought which was sufficiently dilute to yield submaximal deposits, and yet sufficiently concentrated so as to partially repress the action of the inhibitors. Solution 10/0/10 appeared to be suitable and was chosen as the reference system (Table IV). Mg^{++} was included in all media in 1 mM concentration. Studies of the type recorded in Table I were then made in which the degrees of mineralization at 10/7/0, 10/7.5/0, 10/8/0, 10/9/0, and 10/0/10 were compared at 5 and 18 hours. Solution 10/0/10 proved to be approximately equivalent to 10/7.5/0-10/8/0.

Inhibition in the presence of 1 mM cyanide was found to decrease gradually between 10/5/0 and 10/7/0 and more rapidly above 10/7/0. Virtually no inhibition could be detected in solution 10/8/0 after 18 hours of incubation, as was the case with the equivalent organic solution 10/0/10 (Table IV).

Raising the P concentrations also tended to reverse the Mg^{++}-fluoride block in both organic and inorganic phosphate media, but not as readily as with Mg^{++}-cyanide. The degree of calcification in solutions 10/8/0 and 10/0/10 was found to be the same within the limits of error of assessing the poorly defined deposits. In these experiments, as in others involving inhibitors, the degree of inhibition

TABLE III. Magnesium-Dependence of Fluoride Inhibition in β-Glycerophosphate Medium. (10/0/5.5)

Mg^{++} conc., mM/L	Fluoride conc., mM/L	Incubation period, hr	Degree of calcification Test tissue	Control*
0	.1	5	1.1(2+)	2.3(4+)
0	.1	18	2.6(4+)	2.8(4+)
0	1	5	2.5(3+)	2.3(4+)
0	1	18	2.9(4+)†	2.5(4+)†
1	.1	5	0 (0)	1.8(4+)
1	.1	18	0 (0)	2.3(4+)
1	1	5	0 (0)	1.8(4+)
1	1	18	1 (+)	3.1(4+)

* No fluoride present in calcifying solution.
† Identical results obtained with sol. 10/5/0.

TABLE IV. Relief of Cyanide and Fluoride Inhibition at Increased Inorganic and Organic Phosphate Levels. (Ca^{++}) = 2.5 mM, (Mg^{++}) = 1 mM.

Cyanide conc., mM/L	Fluoride conc., mM/L	Incubation time, hr	Inorganic phosphate: Conc., mg % P	Inorganic phosphate: Degree of calcification, Test tissue	Inorganic phosphate: Degree of calcification, Control*	β-Glycerophosphate: Conc., mg % P	β-Glycerophosphate: Degree of calcification, Test tissue	β-Glycerophosphate: Degree of calcification, Control*
1	0	18	7	1.9(3+)	2.6(4+)	10	2.3(4+)	2.7(4+)
1	0	18	8	3.6(4+)	3.7(4+)	10	3.6(4+)	3.7(4+)
0	.1	18	8	~3 (4+)†	3.2(4+)	10	~3 (4+)†	3.3(4+)

* Calcifying solutions devoid of cyanide and fluoride.

† Deposit did not possess the depth or clarity of the control section. Virtually no calcification was noted in a 5-hr incubation period.

became most pronounced with cartilage slices from rats maintained on the rachitogenic diet for more than three weeks.

Role of cations in the inhibition phenomenon. As indicated in this and earlier papers (1,11), cyanide, unlike fluoride, has no influence *per se* on *in vitro* calcification. The common physiological cation Mg^{++} readily mediates the cyanide effect, while the monovalent Na^{+} and K^{+} ions present in calcifying solution appear to have no action. To determine the specificity of the phenomenon, other ions were also tested without regard to their possible biological distribution. The results are given in Table V. Like Mg^{++}, the ions Sr^{++}, Ba^{++}, and Mn^{++} were found to be inhibitory in conjunction with cyanide, but not significantly in its absence at the designated concentrations (incubation period, 18 hours). However, these cations blocked calcification when their concentrations were increased severalfold. Of interest in this connection is the remarkably inhibitory nature of Mn^{++}, which was apparent at a concentration of 0.05 mM per liter = 1/50 of the calcium content of the calcifying solution.

Discussion. It is evident from these studies that cyanide and fluoride ions, in the presence of *magnesium* ions added to the calcifying solutions, inhibit calcification *in vitro* not only with inorganic phosphate(1), but also with β-glycerophosphate as the sole source of phosphate. In the absence of *added magnesium,* cyanide does not inhibit, while 10^{-4} molar fluoride exerts an inhibitory effect in the first few hours which disappears later. This initial inhibition may be due to magnesium originally present in the bone cartilage which diffuses out later. When the concentration of fluoride is 10^{-3} molar the degree of mineralization is actually enhanced, while in the presence of magnesium mineralization is blocked. It must be pointed out in reference to the comments of Marks, *et al.*(2), that magnesium ions in the concentration employed either had no effect or were only mildly inhibitory. The comparison of fluoride or cyanide with magnesium was always made against control bone sections in solutions containing the same concentrations of magnesium.

The nature of our findings with fluoride and cyanide ions in β-glycerophosphate medium requires a reexamination of current theories of calcification. It has been suggested by Gutman and coworkers(5-7) that calcification may proceed along either of 2 completely discrete pathways, depending on the form in which phosphorus is supplied to the mineral solution. The first is assumed to involve phosphorylative glycogenolysis and requires phosphorus in the form of inorganic phosphate or a phosphorylated glycolytic intermediate. The second pathway is thought to involve hydrolysis by bone phosphatase of its typical substrates not occurring in the scheme of glycolysis, such as β-glycerophosphate or phenyl phosphate, with the local liberation of excess inorganic phosphate and its simultaneous deposition as bone salt. The conclusion that inorganic phosphate released by phosphatase action does not enter the glycolytic cycle was based on the failure to demonstrate fluoride inhibition of this second mechanism. Since our findings now indicate that the nature of the action of fluoride or cyanide ions *is* the same in β-glycerophosphate as in inorganic phosphate under comparable experimental conditions, there would appear to be no justification at this time for assuming two such wholly discrete pathways. Indeed,

TABLE V. Effect of Cyanide on Calcification in the Presence of Divalent Cations Other than Magnesium. (Incubation period, 18 hours.)

Cation	Conc., mM/L	Cyanide conc., mM/L	Degree of calcification: 10/5/0 Test tissue	10/5/0 Control*	10/0/5.5 Test tissue	10/0/5.5 Control*
Sr^{++}	1	1	0 (0)	1.6(4+)		
Ba^{++}	.1	1	1.3 (+)	2 (4+)	1(+)	1.8(4+)
	.5	0	0 (0)	2 (4+)†		
Mn^{++}	.01	1	1 (2+)	1.9(4+)		
	.01	1			0 (0)	1.8(4+)
	.05	0	0 (0)	1.8(4+)†		
Co^{++}	.02	1	2 (4+)	2 (4+)		
Ni^{++}	.02	1	2 (4+)	2 (4+)		

* The control calcifying solutions were devoid of cyanide but contained the specified cations, unless otherwise indicated.

† Control calcifying solution devoid of both cyanide and the specified cation.

from recent evidence(12), taken in conjunction with the present paper, it would appear to be doubtful whether any pathway has as yet been decisively established.

The tendency for high concentrations of phosphate to counter or obscure the inhibition by magnesium-fluoride and, particularly, magnesium-cyanide, the weaker couple, is a likely explanation for the apparent discrepancies that have arisen. The indifferent effect of cyanide in organic mineralizing solutions noted in earlier studies may be attributed to the excessive amounts of glycerophosphate (6) or glycerophosphate plus inorganic phosphate(3,4) employed as compared to the control inorganic medium. That the more intensive block by fluoride may be detected in glycerophosphate medium at as high a concentration as 10 mg % P was originally reported by Robison and Rosenheim(4), but subsequently overlooked in later studies in which still higher concentrations of phosphate were employed(6).

It is of considerable interest that the several ions found to mediate cyanide inhibition, including magnesium, strontium, barium, and manganese, are themselves inhibitory at higher concentrations. Possibly they act by interfering with adsorption or diffusion phenomena at the tissue surface, or with growth of the bone lattice. If this is the case, cyanide ion may serve to complex the metal ion at its site of action and increase its inhibitory activity by preventing its release.

Summary. 1. The influence of several ions on *in vitro* calcification in β-glycerophosphate medium has been compared to their effects in inorganic phosphate solution at two phosphate levels which yield comparable degrees of calcification, *viz.*, 10/5/0-10/0/5.5 and 10/8/0-10/0/10.§ Cyanide ion did not interfere in the absence of magnesium. When magnesium was present there was a partial block of calcification at the lower organic phosphate level equivalent to that obtained in the corresponding inorganic solution. At the higher phosphorus levels, 10/8/0-10/0/10, virtually no inhibition by the magnesium-cyanide couple was evident. 2. Calcification in the dilute glycerophosphate solution (10/0/5.5) was blocked by fluoride in the presence of magnesium. In the absence of magnesium, inhibition by 10^{-4} molar fluoride was noted during the first few hours, but not evident on prolonged incubation. Mineral deposition with 10^{-3} molar fluoride in the absence of magnesium exceeded that obtained with the fluoride-free control. Essentially similar results were presented earlier for the equivalent inorganic mineral solution, 10/5/0(1). At higher inorganic and organic phosphorus levels the fluoride block in the presence of magnesium was partially overcome. 3. The data indicate a striking similarity in the response of the calcifying mechanism in both inorganic and β-glycerophosphate media to the action of fluoride or cyanide ions in the presence and absence of magnesium. It therefore appears

questionable whether one may, at this time, associate the calcification process in inorganic phosphate and glycerophosphate solutions with two completely discrete mechanisms. 4. Strontium, barium, and manganous ions share with magnesium the property of mediating the cyanide inhibition of calcification.

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Effects of a Combined Deficiency of Vitamins E and B_6 on Blood Picture of Rat.* (20854)

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One sign of acute vit. E deficiency in monkeys and rabbits is leucocytosis(1,2). There is a marked increase in the numbers of circulating granulocytes with little change in the numbers of blood lymphocytes(1,2). Vitamin B_6 is also concerned in hemopoiesis. It has been shown that B_6-deficient dogs(3) and pigs(4) develop a microcytic anemia. Weir *et al.*(5) reported that the injection of a vit. B_6 antagonist (desoxypyridoxine) into mice resulted in a decrease in circulating lymphocytes and an increase in granulocytes. These changes could be reversed by the administration of pyridoxine. Quite recently Vilter *et al.*(6) reported the effects of administering desoxypyridoxine to man. They observed an absolute lymphopenia with a tendency toward polymorphonuclear leucocytosis. In contrast Wintrobe *et al.*(4) observed no significant change in leucocytes as a result of vit. B_6 deficiency in pigs.

In general, the leucocyte changes which have been observed in vit. B_6 deficiency resemble those of vit. E deficiency. There is some biochemical data which suggest a relationship between vit. E and vit. B_6 in metabolism(7,8). For these reasons a study was made of the effect of a combined deficiency of vit. E and vit. B_6 on the hemogram of the rat.

Methods. Weanling littermate Sprague-Dawley rats of both sexes were fed a basal

TABLE I. Weight Changes and Food Consumption of Rats Fed the Experimental Diets.

Diet	No. of rats	Mean initial wt, g	Mean final wt, g	Mean daily food intake, g
Basal	9	50	102	4.6
" + E	7	49	106	5.4
" + B_6	7	47	194	8.6
" + B_6 + E	7	48	223	8.3

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