cording to Federal Government instructions and a substantial plant expansion program has been undertaken. Of interest in connection with needs for plastics is the program of the multiple fellowship that is engaged in research on polymerizable silicon compounds. The likely uses of these silicon derivatives are many and varied; certain applications have been proposed which have potential utility in warfare. Fellowship research is also being conducted on the employment of plastics in water and gas meters and for many other purposes.

W. A. HAMOR

SPECIAL ARTICLES

PHOSPHORYLATIVE GLYCOGENOLYSIS AND CALCIFICATION IN CARTILAGE[1]

PHOSPHORYLASE, an enzyme which catalyzes the reaction

Glycogen + inorganic phosphate ⇌ glucose-1-phosphate

(the first step in phosphorylative glycogenolysis in muscle and liver),[2] has recently been found in significant concentration in the epiphyses of growing rats and rabbits.[3] This enzyme can initiate conversion of the glycogen of hypertrophic cartilage cells into phosphoric ester substrates for cartilage (bone) phosphatase, and may thus play a significant role in the calcification of cartilage. We have investigated this by determining the effects of known inhibitors of the glycogenolytic cycle in muscle[4] upon *in vitro* calcification of cartilage (Table I).

M/100 phlorizin markedly, in some experiments almost completely, inhibited calcification of cartilage in solutions containing 8 mg per cent. Ca and 5 mg per cent. P as inorganic phosphate. M/200 phlorizin consistently caused partial but definite inhibition, M/2,000 phloretin almost completely blocked calcification. Adding glucose-1-phosphate to these solutions restored the capacity of cartilage to calcify well, even in the presence of phlorizin or phloretin. Since phlorizin and its aglycone, phloretin, principally inhibit phosphorylase activity,[4] these results imply that phosphorylase is not only present, but that its action (the formation of glucose-1-phosphate) is necessary for calcification of cartilage in a medium containing P as inorganic phosphate.

How the P of glucose-1-phosphate is then made available for the formation of bone salts apparently depends upon the activities of 3 enzymes present in calcifying cartilage: phosphorylase, phosphatase and phosphoglucomutase, all of which act upon this ester. Significant dephosphorylation of glucose-1-phosphate by phosphorylase is improbable, at least under the equilibrium conditions of *in vivo* calcification, because the glycogen stores of hypertrophic cartilage do not increase as bone salts are deposited, but disappear. Direct dephosphorylation of much glucose-1-phosphate by phosphatase is also unlikely, for reasons given elsewhere.[3] Equilibrium conditions apparently favor conversion of glucose-1-phosphate by phosphoglucomutase to glucose-6-phosphate and thereafter (by the glycolytic enzyme system known to be present in cartilage),[5] to subsequent phosphoric esters of the glycolytic series. Such, at least, is the implication of observations made by Robison and Rosenheim,[6] which we have confirmed: *in vitro* calcification of cartilage in a medium containing P only as inorganic phosphate is blocked by M/1,000 cyanide, by M/1,000

TABLE I

EFFECT OF INHIBITORS OF GLYCOGENOLYSIS ON *in vitro* CALCIFICATION OF CARTILAGE* WHEN P IS SUPPLIED AS INORGANIC PHOSPHATE, AND AS PHOSPHORIC ESTERS OF THE GLYCOGENOLYTIC SERIES (pH ADJUSTED TO 7.4; TIME 18 HOURS; TEMP. 37°C.)

Phosphoric ester added to calcifying solution†	Inhibitor	Calcification
None (inorganic P only)	none	++++
"	M/100 phlorizin	+
"	M/200 phlorizin	++
"	M/2,000 phloretin	±
Glucose-1-phosphate	M/100 phlorizin	+++
"	M/2,000 phloretin	+++
None (inorganic P only)	M/1,000 KCN	+
"	M/1,000 iodoacetate	+
"	M/10,000 NaF	±
Glucose-1-phosphate	M/1,000 KCN	+
"	M/1,000 iodoacetate	+
"	M/10,000 NaF	±
2-phosphoglycerate	M/100 phlorizin	+++
"	M/1,000 KCN	+++
"	M/1,000 iodoacetate	++++
"	M/10,000 NaF	±
α-glycerophosphate	M/100 phlorizin	+++
"	M/1,000 KCN	+++
"	M/1,000 iodoacetate	++++
"	M/10,000 NaF	++++

* We used the proximal ends of the tibiae of 20–22 day old male rats made somewhat rachitic by 8–12 days maintenance on a Steenbock-Black diet. Each tissue slice was placed in a tube containing 40 cc. control or calcifying solution, the tubes being gently rocked mechanically in a water-bath. The slices were then stained with silver nitrate.

† Solutions contained the control basal salt solution (6) plus 8 mg per cent. Ca as $CaCl_2$, plus 5 mg per cent. P as phosphate buffer, plus (where added) 10 mg per cent. P as phosphoric ester, plus (where added) inhibitor. All concentrations are those in the final solution. pH adjustments were made by choosing suitable phosphate buffers; where phlorizin or KCN were added, it was necessary to make further pH adjustments with $NaHCO_3$ resp. HCl.

[1] Supported by a John A. Hartford Fund Gift. We are indebted also to Dr. D. E. Green for certain phosphoric esters and for many helpful suggestions.

[2] C. F. Cori and G. T. Cori, *Ann. Rev. Biochem.*, 10: 151, 1941.

[3] A. B. Gutman and E. B. Gutman, *Proc. Soc. Exp. Biol. and Med.*, 48: 687, 1941.

[4] J. K. Parnas, "Glykogenolyse," in Handbuch d. Enzymologie, Leipzig, 2: 902, 1940.

[5] G. M. Hills, *Biochem. Jour.*, 34: 1070, 1940.

[6] R. Robison and A. H. Rosenheim, *Biochem. Jour.*, 28: 684, 1934.

idoacetate and by M/10,000 fluoride, concentrations too low to inhibit bone phosphatase or to affect the solubility of bone salts. The inhibition by fluoride, currently believed to prevent formation of phosphopyruvate from 2-phosphoglycerate by blocking the action of enolase, indicates that dephosphorylation by phosphatase does not take place prior to that stage in the cycle. Calcification of cartilage, accordingly, involves phosphorylative glycogenolysis at least to the point of phosphopyruvate formation.

An exacting test of the role of phosphorylative glycogenolysis in this connection can be made by determining the effect of various inhibitors upon *in vitro* calcification of cartilage when P is supplied as the several phosphoric esters of the glycogenolytic series. We find that calcification with glucose-1-phosphate, in concentrations of 10 mg per cent. P, is not inhibited by phlorizin but is inhibited by cyanide, iodoacetate and fluoride; calcification with 2-phosphoglycerate is not inhibited by phlorizin, cyanide or iodoacetate but is by fluoride; calcification with α-glycerophosphate is not markedly inhibited by any of these agents. These results are consistent with what is known of phosphorylative glycogenolysis in muscle.[4] If phosphoric esters not in the series but readily dephosphorylated by bone phosphatase (β-glycerophosphate, phenylphosphate) are used, good calcification is obtained, which can not be blocked by inhibitors of glycogenolysis. If phosphoric esters not in the series and not dephosphorylated by bone phosphatase are used, calcification does not occur.

Conclusions. Calcifying cartilage contains an enzyme system for phosphorylative glycogenolysis. This system constitutes part of Robison's "second mechanism of calcification."

The processes of phosphorylative glycogenolysis are essential for *in vitro* calcification of cartilage in solutions containing P only as inorganic phosphate. They provide a mechanism whereby in such a medium lacking preformed phosphoric ester substrate for bone phosphatase, a concentration of P can be effected locally at the site of calcification in the cartilage matrix.

This mechanism probably operates in normal, *in vivo* calcification of cartilage. It could account for the selective calcification occurring in glycogen-rich primary centers of fetal ossification before erythrocytes, the only adequate source of phosphoric esters hitherto recognized, appear at the site of calcification.

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THE MILK-INFLUENCE OF BREAST TUMORS IN MICE[1]

IN this report we wish to present further data on the characteristics of the active milk-influence which plays an important role in the development of spontaneous carcinoma of the mammary glands in mice.[2,3]

Details of the experiments were as follows. Lactating females of the cancerous A strain were killed on 2/6/41 and their mammary glands removed. The tissue was macerated in a tissue-press and forced through a disc having openings 0.045 of an inch. The macerated material was filtered, using a fine wire cloth and 38 cc of milk, etc., was obtained to which was added an equal amount of sterile physiological salt solution. The extract was filtered through a Seitz filter and the filtrate (called filtrate A) was injected subcutaneously or intraperitoneally into 15 mice of the fostered C3H strain. The incidence of breast tumors in breeding females of the fostered C3H strain is about 2 per cent. The injected animals, as were all the mice used in these studies, were four weeks of age at the time of treatment and each received 2 cc of the filtrate. Nine of the injected mice have developed breast tumors, one has died non-cancerous and the others are living. All the females in this and the following experiments were used as breeders.

To the macerated tissue and the unfiltered residue of filtrate A was added 40 cc of distilled water. The extract secured following filtering with a wire cloth was filtered through the Seitz filter. This filtrate (called filtrate B) was injected, as above, into 12 females of the fostered C3H stock. Ten mice have developed breast tumors.

To determine if the unfiltered material contained the active milk-influence, 17 fostered C3H mice were given, in small dishes, the unfiltered residue and the macerated tissue diluted 1:3 with water. The average amount given per mouse was 7 cc, and most of the material was consumed. Fourteen of these females have developed breast tumors, 2 are living and 1 died non-cancerous.

Mice receiving the filtrates developed tumors at an average age of 287 days and the females receiving the unfiltered extract at 244 days.

On 4/17/41, 75 cc of distilled water was added to 15 cc of macerated breast tumors (tissue-press). The extract was given by mouth to 13 mice of the fostered C3H stock and 6 females of the BAF_1 generation (C57 black ♀ × A ♂, normal incidence of breast tumors, 1 per cent.).[3] The average amount given per female was 3.8 cc. Nine fostered C3H and 1 BAF_1

[1] Assisted by grants from the National Cancer Institute and The Jane Coffin Childs Memorial Fund for Medical Research.

[2] J. J. Bittner, SCIENCE, 84: 162, 1936.

[3] *Idem*, Trans. and Studies of the College of Physicians of Philadelphia, 9: 129, 1941.