rats showed the dermatitis. The general appearance of the rats grayed by the two methods is quite different.

The results of our experiments indicate that graying of the hair may be produced by a positive factor in the diet as well as by the lack of some factor, such as pantothenic acid. It is possible that the phenylthiocarbamide may combine with the pantothenic acid or some other factor in the body and thus produce an actual deficiency of the latter.

These experiments bring more evidence for the close relationship between graying of the hair and the activity of the thyroid gland. However, the fact that graying was found in several animals in which supposedly all of the thyroid gland had been removed indicates that the phenylthiocarbamide probably has a more direct effect on the hair.

Summary. (1) After being placed on a stock diet and given phenylthiocarbamide solution as the sole source of fluid, with one exception 23 black or black hooded rats showed definite graying, some as early as 27 days and all within 58 days. (2) Approximately 83 days after the phenylthiocarbamide solution was replaced by tap water, five rats that had shown marked graying turned completely black again. (3) The experiments showed that graying may result from a positive factor in the diet, a poison, as well as from a deficiency of some factor.

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A Phosphorylase in Calcifying Cartilage.*

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It has been shown by histochemical[1, 2, 3] and chemical[3, 4] methods that cartilage cells preparatory to calcification accumulate large stores

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1 Creighton, C., *Microscopic Researches on the Formative Property of Glycogen*, Adam and Charles Black, London, 1896.

2 Harris, H. A., *Nature*, 1932, **130**, 996.

3 Glock, G. E., *J. Physiol.*, 1940, **98**, 1.

4 Hoffman, A., Lehmann, G., and Wertheimer, E., *Arch. f. d. ges. Physiol.*, 1928, **220**, 183.

of glycogen, which disappear abruptly just prior to or during the early course of calcium deposition. The processes of glycogen depletion and of calcification appear to be closely coördinated, suggesting some inter-relation;[1, 2] for example, phosphoric esters formed during glycogen breakdown might serve as substrates for bone phosphatase.[2, 3, 4, 5] This inherently plausible hypothesis, as yet unsupported by direct evidence, has been largely ignored in current speculation concerning the mechanisms of calcification.

In liver and muscle, according to recent studies by Cori,[6] Parnas[7] and others, the major pathway of glycogen degradation involves phosphorolysis and the formation of intermediate phosphoric esters. The glycogenolytic cycle is initiated by the reaction

Glycogen + inorganic phosphate ⇌ glucose-1-phosphate

which is catalyzed by an enzyme, phosphorylase, shown to be present in liver, muscle, and certain other tissues. Evidence is offered here that a similar phosphorylating enzyme system, associating glycogenolysis with calcification, occurs in calcifying cartilage.

Methods. The proximal and distal ends of the femora, tibiae and humeri of young rabbits (500-800 g) or rats (20-25 days) were used, after careful removal of all muscle and most shaft bone. The tissue was rapidly weighed, minced and ground, then transferred to glass-stoppered centrifuge tubes. These, in experiments with tissue pulp, contained a 1:1 proportion of digest mixture prepared as indicated in Table I (1.0 cc was used where the tissue weighed less than 1.0 g), with which the pulp was quickly stirred. After brief centrifugation, duplicate control samples were removed from the supernatant with a Lang-Levy micropipette. The digest was then mixed and allowed to stand, samples being taken at intervals after preliminary centrifugation.

In experiments in which not tissue pulp but tissue extracts were used, the centrifuge tubes contained only distilled water equal in volume to the tissue used, but not less than 1.0 cc. After stirring 15 minutes, then centrifuging, the supernatant extract was added to digest mixtures (prepared so as to give proper final concentrations), as described.

Serial samples obtained at intervals during digestion were discharged into 1.0 cc 5% trichloracetic acid. After standing 10 minutes, 9.0 cc water was added, and the inorganic P content of filtrate

[5] Robison, R., and Rosenheim, A. H., *Biochem. J.*, 1934, **28**, 684.

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

[7] Parnas, J. K., *Glykogenolyse, Handbuch d. Enzymologie* (edited by Nord and Weidenhagen), Akad. Verlagsgesellschaft, Leipzig, 1940, **2**, 902.

aliquots determined by the Kuttner-Lichtenstein method,[8] using an Evelyn photoelectric colorimeter. The percentages of inorganic P and reducing sugar recoverable after hydrolysis of digest filtrates were determined in some samples after boiling with N sulfuric acid for 7 and 30 minutes.

As indicated in the accompanying tables, application of this semi-microtechnic to liver pulp and extracts yields results in satisfactory agreement with those recorded for liver by Ostern, Herbert and Holmes[9] and by Cori, Cori and Schmidt.[10]

Results. When suitable concentrations of glycogen and inorganic P were added to ground epiphyses of young animals, the inorganic P content of the digest decreased rapidly (Table I). We did not identify the phosphoric esters formed beyond establishing that no

TABLE I.

Effect of Young Rabbit Liver and Epiphyseal Pulp on Inorganic P Content of Phosphate-glycogen Mixtures; Influence of Temperature and Fluoride on the Reaction.

Exp.	Additions* to tissue pulp	Temp., °C	Time, (min.)	Liver pulp Inorg. P		Epiphyses pulp Inorg. P	
				mg/g tissue	% change	mg/g tissue	% change
1	P,G,F	37	2	1.62	0	1.13	0
			15	0.54	—67	0.85	—25
			30	0.47	—71	0.75	—34
			60	0.38	—76	0.61	—46
			120	0.52	—68	0.57	—50
2a	P,G,F,	22	2	1.38	0	1.04	0
			15	0.74	—50	0.84	—19
			30	0.45	—67	0.74	—29
			60	0.22	—84	0.64	—38
			120	0.20	—85	0.62	—40
2b	P,G	22	2	1.52	0	1.03	0
			15	1.18	—22	0.83	—19
			30	1.16	—24	0.73	—29
			60	1.14	—25	0.68	—34
			120	1.37	—10	0.59	—43
3	P,G,F	15	2	1.46	0	1.09	0
			15	1.03	—29	0.96	—12
			30	0.74	—49	0.91	—17
			60	0.41	—72	0.87	—19
			120	0.28	—81	0.84	—23

*P—M/45 phosphate buffer at pH 7.2.
G—0.4% glycogen.
F—M/40 NaF.
(All concentrations indicated signify those in final digest mixture.)

[8] Bodansky, A., *J. Biol. Chem.*, 1932, **99**, 197.

[9] Ostern, P., Herbert, D., and Holmes, E., *Biochem. J.*, 1939, **33**, 1858.

[10] Cori, G. T., Cori, C. F., and Schmidt, G., *J. Biol. Chem.*, 1939, **129**, 629.

significant inorganic P could be recovered after 7 minutes' hydrolysis, even in the presence of fluoride, and that a variable amount, sometimes over 50%, reappeared after 30 minutes' hydrolysis.

Esterification was less marked than observed with liver pulp but the activity of the two tissues in relation to time and temperature was similar (Table I). As with liver, the disappearance of inorganic P was inhibited by M/100 phlorizin. A striking difference, however, was observed with M/40 NaF which, in contrast with its marked effect on liver, caused no change in the rate of disappearance of inorganic P by ground epiphyses (Table I).

Whereas with tissue pulp, addition of muscle adenylic acid was found to be unnecessary, aqueous extracts of the epiphyses of young animals showed no phosphorylase activity in the presence of glycogen and inorganic P unless or until the coenzyme was added (Table II). This behavior corresponds with that recorded for liver and muscle phosphorylase.[9, 10]

When glucose-1-phosphate was added to aqueous extracts of young epiphyses in the presence of catalytic amounts of glycogen and muscle adenylic acid, an increase in the inorganic P content of the digest was observed (Table II). An iodine test for glycogen after several hours gave a faint brownish color which gradually faded.

Discussion. The representative results cited were not obtained with isolated hypertrophic cartilage but with whole epiphyses, which

TABLE II.

Effect of Aqueous Extracts of Young Rabbit Epiphyses on Inorganic P Content of Phosphate-glycogen Mixtures, with and without Added Muscle Adenylic Acid; and on Glucose-1-phosphate. (Temp. 22°C.)

Exp.	Additions to tissue extracts	Time (min.)	Inorganic P	
			mg/g tissue	% change
4a	M/25 phosphate buffer pH 7.2, 0.5% glycogen, M/25 NaF and muscle adenylic acid	2	.83	0
		15	.67	—19
		30	.64	—23
		60	.61	—27
		120	.64	—23
4b	Same but adenylic acid omitted	2	1.19	0
		15	1.18	0
		30	1.18	0
		60	1.19	0
		120	1.17	0
4c	Adenylic acid now added to 4b	15	.69	—39
5	M/200 glucose-1-phosphate, traces of glycogen and adenylic acid	1	.18	
		30	.41	
		60	.58	
		240	1.52	

include bone, marrow, blood, small-cell cartilage, etc. Part of the phosphorylase activity noted could be ascribed to the hypertrophic cartilage present, however, since this, when dissected out as cleanly as possible from young rat epiphyses, produced a 17% decrease in the inorganic P content of phosphate-glycogen digests in one hour. The epiphyses of adult rats, the persistent plate of which contains only small-cell cartilage, also show definite phosphorylase activity although articular (non-calcifying) cartilage has no significant effect, according to Lutwak-Mann.[11] This may be due, in part, to the presence of osseous tissue since we found distinct activity in isolated cortical bone from the shaft of both young and mature rats.

The results cited in Tables I and II imply a very rapid rate of reaction of the enzymes involved. This contrasts with the extremely slow reverse catalytic effect of bone phosphatase. In fact, the dephosphorylating action of aqueous extracts of young rabbit epiphyses was found to be slight at pH 7.2 and 22° : 0.8 mg inorganic P was split off from β-glycerophosphate substrate whereas 19.4 mg were hydrolyzed by the same extract at pH 8.6 and 37°. Activation by adenylic acid further indicates that our results cannot be ascribed wholly to bone phosphatase.

We conclude that there appears to be present in calcifying cartilage a phosphorylative glycogenolytic enzyme system capable of synthesizing potential substrates for bone phosphatase at zones of calcification before blood sources become available, and for supplementing those blood sources after they become available. Inorganic phosphate for calcification can be formed from organic esters not only by bone phosphatase but by phosphorylase.

If glycogenolysis is a prerequisite for calcification in cartilage, it becomes clear why calcification *in vitro* is blocked by glucose, fluoride and iodoacetate[5] in amounts too small to inhibit bone phosphatase or to affect the solubility of bone salts. The inhibitory effects of glucose, fluoride and iodoacetate further make it unlikely that dephosphorylation of glucose-l-phosphate by bone phosphatase (which we find takes place readily *in vitro* at pH 8.6) affords the chief source of inorganic P for calcification; moreover, glucose-l-phosphate appears to be converted to other phosphoric esters too rapidly to serve as the chief substrate for bone phosphatase.

[11] Lutwak-Mann, C., *Biochem. J.*, 1940, **34**, 517.