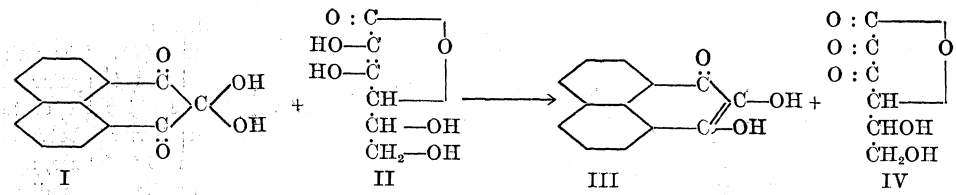

I II III IV

The proportionality of the color obtained in this reaction is in good agreement with Beer's law.

We have used this reaction in the estimation of ascorbic acid through spectrographic determination of dihydroxy-peri-naphthindone produced by reduction of the reagent by ascorbic acid in pure solution. The amount of the reduction product thus produced is estimated by the intensity of absorption at 475 mμ; the excess of the reagent, which should always be present, does not absorb at this region of the spectrum. This is not true if estimation is carried out at 345 mμ, and higher values are obtained. However, if just enough reagent is added to react completely with the known amount of ascorbic acid present, good results have been obtained.

The nature of this reaction is illustrated by the following experiment. Eleven milligrams of pure ascorbic acid is weighed, dissolved, and completed to 100 ml with ethyl alcohol. To 5 ml of this solution is added one ml of peri-naphthindanetrione hydrate in alcohol (2 mg/one ml). These are mixed thoroughly and stoppered at room temperature. The color gradually develops and reaches its maximum intensity after 10 min, when it is ready for estimation. The stability of the color permits its measurement with ease at any time from 10 min to 24 hr after the reagents are mixed. A cell of 5-mm thickness is filled with this solution; the control cell is filled with pure ethyl alcohol and is estimated, using a Hilger Barfit quartz spectrograph.

For pure dihydroxy-peri-naphthindone the two bands, one being in the ultraviolet and the other in the visible part of the spectrum, have intensities which can be expressed as $E^{1\%}_{1\,cm}$ 345 = 525 and $E^{1\%}_{1\,cm}$ 475 = 150.

This reagent, apart from the fact that it is easily prepared, inexpensive, and stable, is specific. To a solution of peri-naphthindanetrione hydrate a solution of the possible interfering substances in plant, animal, and biological media was added under the same experimental conditions used in the case of ascorbic acid, and no color was developed. The following substances were tested: glucose, fructose, alanine, leucine, isoleucine, phenylalanine, lactic acid, aceto-acetic acid, pyruvic acid, urea uric acid, acetone, and dehydroascorbic acid.

Details of this method will be published elsewhere.

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A Method for Evaluating the Relation of Glycogen to Inorganic Salt Deposition in Surviving Cartilage Slices *in Vitro*[1]

Paul A. Marks[2] and Ephraim Shorr

The Russell Sage Institute of Pathology,
the Department of Medicine,
Cornell University Medical College and
The New York Hospital, New York City

Glycogen has been known to be present in the hypertrophic cartilage cell for almost a century (*1*), and its relation to the ensuing ossification of the cartilage was suggested by Creighton (*2*) as early as 1896. The evidence that has accumulated since then has been, for the most part, favorable to the concept that a close relationship exists between the region of glycogen accumulation in cartilage prior to calcification and the area of subsequent lime salt deposition (*3*, *4*).

Harris (*5*) suggested that the role of glycogen was to furnish phosphate ester substrate, possibly for the phosphatase found to be present in cartilage by Robison (*6*). More specific evidence of the relation of glycogen to calcification was provided by Gutman and his associates, who demonstrated phosphorylase activity in cartilage (*7*), as well as the role of phosphorylative mechanisms in the deposition of calcium in cartilage *in vitro* (*8*). This latter observation has been confirmed in our laboratory and found to apply to strontium as well (*9*). Interest in strontium grew out of experiments in man which suggest that strontium can supplement calcium to cause a more rapid remineralization of the skeleton in osteoporosis than usually occurs with calcium alone (*10*).

Previous histochemical studies dealing with the relation between glycogen and calcification of cartilage have utilized techniques which involved a comparison of the glycogen-staining areas in one piece of cartilage with the calcified portion in another (*4*). It is the purpose of this note to describe a method by which a direct correlation can be made in the same cartilage slice between the glycogen zone and the area of subsequent lime salt deposition. The procedures involved are simpler than those required for the staining of glycogen in fixed sections, and the method is particularly applicable to the analysis, in surviving cartilage slices, of the enzymatic reactions involved in the process of calcification.

The details are as follows: The proximal ends of the tibiae and the distal ends of the femurs of mildly rachitic

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[2] U. S. Public Health Service postdoctorate research fellow.

TABLE 1

EFFECT OF PREINCUBATION OF RACHITIC CARTILAGE IN BASAL SOLUTION ON GLYCOGEN CONTENT AND SUBSEQUENT CALCIUM AND STRONTIUM DEPOSITION *in vitro*

Zone	No. of experiments*	Controls		Experimental	
		Fresh bone slice	After incubation in nutrient media†	After incubation in basal solution	After incubation in basal solution; then incubation in nutrient media
Glycogen-staining area	32	+++	...	0	...
Calcium deposition	16	...	++++	...	0
Strontium deposition	16	...	++	...	0

* Four bone slices per experiment. All incubation periods were for 18 hr at 37.5° C.

† Calcifying or strontifying media.

rats are dissected free of soft tissue, and sliced longitudinally. The freshly dissected bone slices are stained for glycogen by exposing them to the vapors of Lugol's solution for 15 min at a distance of 0.5 cm from the surface of the solution. The bone slices are then examined under 80× magnification, and the extent of the glycogen-staining area is noted. They are then placed in a 0.1 per cent KI solution for 10 min to remove the iodine. Complete removal of the iodine is essential to avoid its interference with the subsequent deposition of inorganic salts. The above procedure accomplishes this, as far as could be detected by the reexamination of the slices under 200× magnification. The bone slices are washed free of KI in 2 changes of a noncalcifying modified Ringer-bicarbonate solution containing NaCl 70 meq, KCl 5 meq, $NaHCO_3$ 22 meq per liter. They are then incubated for 18 hr at 37.5° C in the above medium, to which phosphate and calcium or strontium are added in appropriate concentrations for inorganic salt deposition (*8, 9*). The incubation is carried out in a Warburg bath, with gentle shaking, in stoppered 25-ml Erlenmeyer flasks containing 10 ml of the incubation medium. Throughout the incubation period the medium is in equilibrium with a mixture of 5% CO_2–95% O_2 in the gas space and thereby is maintained at a constant pH of 7.4. Following incubation, the bone slices are stained by a silver nitrate technique (*8*) to reveal the newly deposited inorganic salts, and placed in glycerol in order to clear the specimen and preserve the silver stain. Under 80× magnification, a direct comparison can be made between the area of inorganic salt deposition and that previously found to contain glycogen.

Iodine does not stain glycogen exclusively (*11*). With the technique described above, the glycogen-containing areas are stained a reddish brown and the remainder of the epiphyseal cartilage takes on a diffuse yellowish hue. The differences in the staining properties are sufficient, however, to permit the ready differentiation and demarcation of the glycogen-containing areas at 80× magnification.

Utilizing this procedure prior to incubation *in vitro*, glycogen was found to be present in the zone of hypertrophic cartilage cells, and to be undetectable in the zone of cells nearest the primary spongiosa, as well as in the resting cartilage cell zone. There was a close correlation between the glycogen-containing area and the region in which both calcium and strontium were deposited after incubation *in vitro*. However, the deposition of inorganic salts tended to extend slightly below the lower border of the area in which glycogen could be visualized by the technique described.

In order to determine more directly than in previous studies the relation between variations in glycogen content and the ability of cartilage to deposit inorganic salts *in vitro*, the following experiments were carried out.

In the first group (Table 1) bone slices were stained for glycogen prior to and following 18 hr of incubation in the basal solution described above, containing no calcium, strontium, or phosphate. This period of incubation resulted in the complete disappearance of the glycogen-staining areas. When such bone slices were then reincubated in a calcifying or strontifying solution for an additional 18 hr, no deposition of strontium or calcium occurred.

In the second group of experiments (Table 2) bone slices were stained for glycogen prior to and following a 45-min exposure to saliva (as a source of amylase), with changes of saliva every 10 min. This procedure led to a marked diminution in the glycogen-staining area. On subsequent incubation of the bone slices in a calcifying or strontifying medium, the extent of deposition

TABLE 2

EFFECT OF SALIVA (AS A SOURCE OF AMYLASE) ON GLYCOGEN CONTENT AND SUBSEQUENT CALCIUM AND STRONTIUM DEPOSITION IN RACHITIC CARTILAGE *in vitro*

Zone	No. of experiments*	Controls		Experimental		
		Fresh bone slice	After incubation in nutrient media†	After incubation in saliva	After incubation in saliva; then incubation in nutrient media.	After incubation in saliva; then incubation in nutrient media + glucose–1–PO_4
Glycogen-staining area	32	+++	...	+	...	...
Calcium deposition	20	...	++++	...	+	+++
Strontium deposition	12	...	++	...	0	+++

* Four bone slices per experiment. All incubation periods were for 18 hr at 37.5° C.

† Calcifying or strontifying media.

of inorganic salts was markedly less than occurred in the control slices. However, if the bone slices, after exposure to saliva, were incubated in a calcifying or a strontifying medium to which glucose-1-phosphate was added, inorganic salt deposition comparable in degree to that in the control slices did occur.

It is evident that the removal of glycogen from cartilage prior to its exposure to a calcifying or strontifying solution markedly interferes with the deposition of these cations. These observations provide additional evidence of the importance of glycogen in the preparatory stages of inorganic salt deposition in cartilage *in vitro*. The influence of the addition of glucose-1-phosphate is consistent with its position in the phosphorylative glycolytic cycle.

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Plant-Growth Inhibitors from Red Kidney Bean Seeds

S. M. Siegel[1]

Department of Botany,
University of Chicago, Chicago, Illinois

In a previous publication (*1*), the writer reported the presence of photolabile germination and growth inhibitors in seeds of the red kidney bean (*Phaseolus vulgaris*). It was maintained at that time that only aqueous preparations were active and that extracts of the whole bean possessed the greatest inhibitory activity. The work reported here includes further investigation of seed coat extracts only. Somewhat different extraction techniques have yielded ether fractions containing inhibitory substances.

As before, the test object employed was the root or root and hypocotyl of the flax seedling. Flax seeds were germinated and grown in Petri dishes on filter paper; this substratum was moistened with aqueous extracts, or with water, after ether preparations had been evaporated to dryness on them. At the end of an incubation period of 45–50 hr, the length of the emerged root or of the root and hypocotyl was measured. Incubation temperature ranged from 24° to 27° C.

TABLE 1

EFFECT OF SEED COAT EXTRACTS ON THE EMERGENCE OF FLAX* (MM)

	Control	Skelly	Ether P	Ether Y	Aqueous
Length of root plus hypocotyl	10.5	10.8	3.9	7.8	6.0
Percentage of control	100	103	37	74	57
Density at 320 mμ	....	....	0.600	0.600	...
Density at 585 mμ	....	....	0.179	0.055	...

* Based on 30–50 seedlings incubated 48 hr with 3 ml of extract; extracts in organic solvents were dried on the filter paper, and the 3 ml of water then added.

Whereas in previous work attempts to extract dry seed coats with ether failed to disclose inhibitors, extraction with ether in the presence of water yielded clear, purple ether fractions with activity. Extracts were obtained as follows: Bean seeds were soaked in water at room temperature for no more than 3–4 hr to soften seed coats; these were then removed. For extract P-3, 10 g of dry seed coats, 30 ml of water, and 30 ml of diethyl ether were placed in 125-ml Erlenmeyer flasks and kept in darkness, with occasional shaking, for about 12 hr. The ether fraction, by this time a deep, clear purple, was separated, shaken 2–4 times with the red-orange aqueous phase, and dried over anhydrous sodium sulfate. Extracts were stored in darkness until use. All operations were conducted in darkness or in diffuse light that did not exceed 15 ft-c intensity. Extract P-4 was prepared using 10 ml of water, 50 ml of ether, and an 8-hr extraction period; continuous agitation was maintained, using a reciprocal shaker. By this method, 100 g of seed coat was extracted, and the ether fractions were concentrated *in vacuo*; the concentrate was dried at 5° C over sulfuric acid.

Other modifications of the techniques described have been successfully employed. Using a Beckmann Model DU spectrophotometer, absorption spectra of the ether extracts were studied for several reasons: (1) once having established the characteristic absorption for the first extract prepared, the presence of the same absorption peaks (Table 1) in subsequent preparations served as a check on the extraction method; (2) it was desirable to find if destruction of the purple pigment in the extract (through the agencies of light, alkali, etc.) was correlated with changes in the inhibitory properties of the preparation.

The relative activities of several preparations are given in Table 1. The greatest activity was found in the purple ether fraction (Ether P); shaking this preparation with sodium hydroxide pellets transformed the extract to a yellow color and diminished its activity by a factor of two (Ether Y).

[1] Present address: Biological Department, Camp Detrick, Md.