Mature rabbits given 60 mg/kilo body weight or more at weekly intervals died; an immature rabbit died after repeated weekly treatments of approximately 50 mg/kilo body weight. Younger animals generally were more susceptible to insecticide poisoning than older ones. Rats given approximately 75 mg/kilo body weight were not affected after repeated weekly treatments. Those given 150 mg/kilo body weight died after repeated weekly treatments. This would seem to indicate that the lowest toxicity for rats falls between these 2 concentrations, considerably above the amount causing death in rabbits. The accumulation of Dieldrin in the organs of the animals treated indicates that repeated treatments may be potentially dangerous.

Conclusions. 1. Dieldrin produced chronic toxicity in rabbits when it was applied once a week at 70 mg/kilo body weight. Less than 30 mg/kilo produced no symptoms. 2. Only 2 of 11 rats dipped in .125, .187, and .250% Dieldrin spray solutions died. 3. Dieldrin produced chronic toxicity in mature rabbits when fed orally approximately 60 mg/kilo body weight. Similar feeding of approximately 150 mg/kilo body weight was fatal to rats. 4. Liver and kidneys of all animals treated and analyzed were found to contain Dieldrin. The amount recovered in these organs was generally in proportion to the amount fed per week, not the duration of feeding. 5. Analyses of milk from cows sprayed with Dieldrin for external parasite control indicated a maximum of 5.9 p.p.m., which was gradually decreased regardless of continued application.

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Calcification VI. Adenosinetriphosphate Content of Preosseous Cartilage.* (19337)

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Enzymes acting on organic phosphate(1,2), particularly those involved in phosphorylative glycogenolysis(2,3), have received serious attention among the "local factors" proposed as being responsible for initiating the process of mineralization. While the existence of glycogen(2-4) and presumably some of the enzymes in the cycle has been indicated(2) the presence of adenosinetriphosphate (ATP), required in such a cycle has not been demonstrated. Cartier(5,6), however, has indicated that if ATP were present it might considerably enhance the degree of mineralization. He reported that added ATP greatly enhances *in vitro* calcification of embryonic sheep bone cartilage.

The purpose of the present investigations was to establish the existence of ATP in bone cartilage. As will be shown in this paper, ATP does exist in significant amounts in the bone cartilage of the rat; furthermore, there appears to be a correlation between the preservation of the calcifying mechanism by calcium and the ATP level.

Procedure. Lutwak-Mann(7) was not able to detect either acid hydrolyzable phosphorus nor significant quantities of other forms of organic phosphorus in trichloracetic acid extracts of articular cartilage. This must be related to the fact that the inorganic phosphorus content of cartilage is very high as compared to the organic phosphorus. It is apparent that if organic phosphorus is to be detected, methods other than those heretofore employed must be used. We decided to use the enzymatic procedure developed by one of

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TABLE I.

bone sections placed in 3 cc cold 10% TCA, let stand 30 min in cold. Centrifuge

- add 3 cc 5% TCA, stand 15 min. Centrifuge
  - dissolved in 2 cc 30% KOH heat in boiling water bath, cool. Add 1 vol 95% alcohol, stand overnight on ice. Centrifuge
    - glycogen wash with 95% alcohol. Centrifuge — discard
      - glycogen, hydrolyze in 2 cc 1 N HCl for 1 hr in boiling water bath. Neutralize to phenolphthalein with NaOH make to vol. Do Nelson glucose determination — discard
  - combine, neutralize, add ½ cc 25% $BaAC_2$ and 1 vol 95% alcohol, stand overnight on ice. Centrifuge
    - dissolve in 3 cc water and 2-3 drops 2 N HCl put through amberlite column (activated with Na) containing 2½ g of amberlite. Check for complete removal of barium, neutralize, make to volume for Deaminase determination (fraction A). — discard

us for the detection of ATP(8). In the first experiments carried out to detect ATP, approximately 6-week-old animals, both on normal and rachitic diets, were anesthetized with nembutal (5 mg/100 g of body weight), the tibiae exposed, and sections of the heads removed. Tissue from 2-6 animals was homogenized, in 3 ml of cold 10% trichloracetic acid (TCA), and filtrates prepared in accordance with the scheme shown in Table I. In later experiments, as indicated in Table I, the sections were soaked in cold TCA for longer periods of time without homogenization. Results obtained with the soaking technics were not significantly different from those after homogenization with respect to ATP, but gave much lower levels of inorganic phosphorus. In the experiments on calcification which are reported here, tissue sections from each animal were split among two groups and placed on glass hooks (2-3 sections on a hook) in Erlenmeyer flasks to which the incubating solutions were added(9). The hooks were made from capillary tubing, the ends of which were inserted into holes bored in paraffined cork stoppers. This was done so that individual variations among different animals could be avoided and insured both sides of the sections coming into contact with the solution instead of only one as in the case of sections placed at the bottom of the flasks. Experiments were also carried out on the glycogen content of the slices after different periods of incubation. Glycogen was precipitated according to the method described in Umbreit, Burris, and Stauffer(10). Glucose was measured by the method of Nelson(11). In a few runs, glycogen was estimated by the procedure of van Wagtendonk(12).

Results and discussions. Ultraviolet spectra

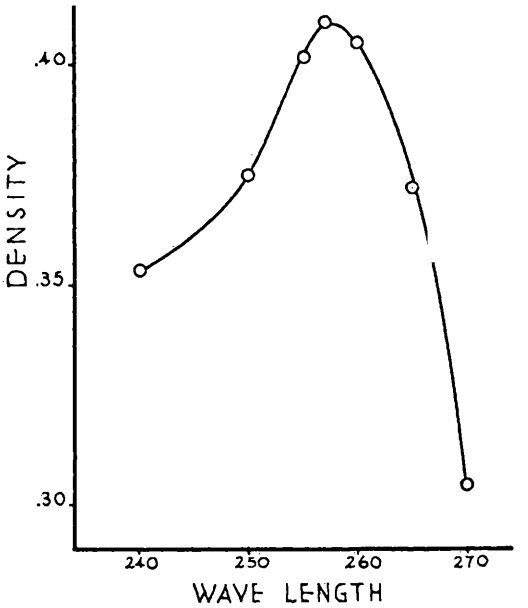

FIG. 1. Absorption spectrum of fraction (A) (see Table I) prepared from epiphyseal cartilage.

TABLE II. ATP Content of Cartilage from Normal and Rachitic Rats. (ATP expressed as adenine. The value per rat represents both tibiae.)

Exp. No.	ATP	No. rats	γ/rat	Condition*
1	34.5	2	17.2	R
2	22.5	2	11.2	R
3	28.5	3	9.2	N
4	58	5	11.6	R
5	59	6	9.8	R
6	47.4	4	11.8	N
7	66.6	6	11.1	R

* R = rachitic; N = normal.

obtained from filtrates prepared as described in Table I (fraction A) have absorption maxima characteristic of the adenine nucleotides with a slight shift towards the lower wave lengths (Fig. 1). Enzyme determinations in addition showed that ATP was present. AMP and ADP were virtually absent. The results of the first group of runs on both normal and rachitic animals are shown in Table II. It is clear from these results that cartilage contains clearly measurable quantities of ATP. (Comparative data on the relative content of cartilage as compared to other tissues will be presented below.)

The next group of studies was concerned with changes in ATP levels in the basal solution used in studies of calcification *in vitro* (13). This solution is free of calcium and phosphate. Two groups of experiments were carried out, one in which the sections were incubated in the basal solution and the other in which the basal solution was supplemented with calcium chloride (2.5 millimoles per liter). Data from a typical experiment are shown in Table III. It is clear that in the early intervals the ATP levels are depleted more rapidly from sections placed in basal solution than from sections placed in basal solution containing Ca^{++} ions. These results are interesting in the light of the observations that calcium ions in the basal solution exert a protective action against deterioration of the calcifying mechanism(9,14). These data suggest that there may be a relationship between the ATP levels and the ability to calcify.

In other studies carried out by Marks and Shorr(3), a correlation was established between glycogen content (as measured by iodine staining) and subsequent ability of cartilage sections to calcify. We measured glycogen levels in the TCA residues of the slices on which we carried out the ATP analyses. Approximately a 50% reduction in the glycogen content of both sets of sections occurred by the end of 12 hours. In contrast to the results obtained with ATP, however, we could find no significant differences between the glycogen content of slices incubated in basal solutions or solution supplied with Ca^{++} (Table III). Since our analytical procedure depends upon the conversion of glycogen to glucose, it was possible that what we were measuring was reducing sugar derived from some other source, for example, from chondroitin sulfate, a common constituent of cartilage. We ruled out this possibility in the following way: chemical determination of glycogen directly using the procedure of van Wagtendonk(12) gave results of the same order of magnitude as that obtained after hydrolysis. If anything, the results were higher. Chondroitin sulfate† in addition did not precipitate with glycogen under our experimental conditions. These conclusions do not imply that glycogen is not involved in calcification, but indicate that under the conditions of our experiment, ATP, rather than glycogen, was probably limiting. Should further investigation indicate that the ATP is in the matrix, one of the difficulties with the theory of Robison(15) as modified by Robison and Rosenheim(1) would be eliminated. This theory visualizes the deposition of bone salt in the following manner:

$$\text{Organic } PO_4 \xrightarrow{\text{phosphatase}} R + PO_4 \xrightarrow[\text{Ca ion}]{\text{2nd mechanism}} \text{Bone salt}$$

The major difficulty with the theory was that hitherto no appreciable amounts of organic phosphate were found in the matrix. This relative lack of organic phosphate is what led Gutman and his coworkers(2,16,17) to investigate glycogen and phosphorylative glycogenolysis in relation to the calcifying mechanism as a source of readily renewable organic phosphate.

† Obtained through the courtesy of Dr. Saul Roseman, Department of Pediatrics and Biochemistry, University of Chicago.

TABLE III. ATP and Glycogen Content of Slices Incubated on Glass Hooks in Basal and Calcium Chloride (2.5 mMols per liter) Supplemented Solutions for Varying Periods of Time.

	Basal Ca			Basal sol.			mg % glycogen	
Time, hr	mg % ad as AA	mg % ad as ADP	mg % ad as ATP	mg % ad as AA	mg % ad as ADP	mg % ad as ATP	Basal sol., Ca	Basal sol.
0	.0	1.1	8.3	.0	1.1	8.3	349	349
3	.3	.9	5.4	.0	1.8	1.6	291	226
6	.0	.9	3.8	.7	.8	.9	219	206
12	.7	.4	2.2	.7	.4		185	188

One last point of interest deserves mention. Expressed as adenine, the ATP level of cartilage is approximately 9 mg %. Expressed as ATP, this would correspond to about 35 mg %. ATP values obtained for liver, kidney, brain and muscle in our laboratory were respectively 40, 29, 50 and 300 mg %. Cartilage therefore has an ATP level in the same range as kidney, liver, and brain.

Summary. ATP has been identified in TCA filtrates of epiphyseal cartilage of rat tibiae. The levels present are approximately those found in kidney, liver, and brain. The disappearance of ATP from cartilage slices incubated in basal solution is retarded initially by small amount of calcium ion. This may account in part for the prevention by calcium of the deterioration of the calcifying mechanism as reported earlier(14). A similar correlation does not occur in the case of glycogen.

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