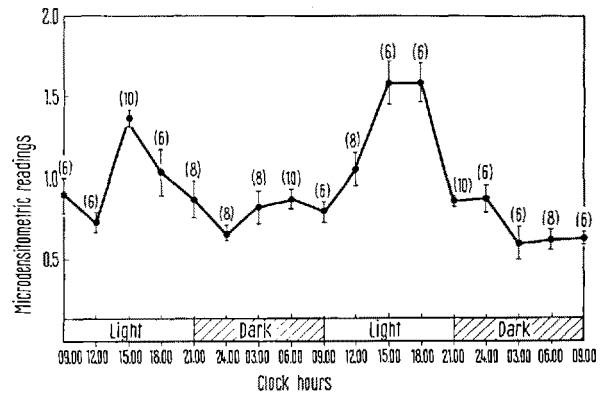
Daily Rhythm of S^{32} Incorporation into Epiphyseal Cartilage in Mice[1]

High resolution autoradiographic techniques with S^{35} have demonstrated that cartilage cells synthesize the chondroitin sulfate of the ground substance of cartilage matrix[2]. Intracellular concentrations of S^{35} may be detected as long as 24 h after injection as the initially labeled cells are maturing, and some tracer begins to appear over the cartilage matrix owing to cellular secretion 48–72 h after injection. It is probable also that the protein and polysaccharide moieties of the matrix are formed concurrently. We have previously shown in rats[3] that the uptake of S^{35} by epiphyseal cartilage continues more actively during the first 12 h following i.v. injection at 09.00 than at 21.00. These preliminary experiments suggested that the differences in uptake were related to variations in cellular activity. The present study in mice was designed in part to confirm these observations and to establish the clock hours of maximum and minimum cartilage matrix synthesis.

Method. CF_1 albino mice, 20–30 g body weight and 3–4 weeks old, were placed in cages of 5 animals each and maintained for 12–13 days under a photoperiod of 12 h (09.00–21.00) alternating with 12 h of darkness (21.00 to 09.00). Food and water were supplied ad libitum. Because the experiment was conducted over a 48 h period, it was desirable to limit the amount of intentional disturbance. Consequently, the animals were actually housed in 2 rooms and they were attended daily from 10.00–11.00. Following the 12–13 day conditioning period, groups of 4–5 mice were injected i.p. with 30 μCi S^{35}-sulfate in 0.2 ml saline at intervals of 3 h over a total period of 2 days. The experiment was begun at 09.00. The injections during the dark environmental period were performed by moving the cages close to the door of the animal quarters so that only a small amount of light entered the room. However, it is conceivable that even a brief exposure to 'dusk' conditions could affect the established photoperiod adversely. Similarly, one might expect changes owing to the necessity to enter the rooms frequently during the actual experimental period. The groups of mice were sacrificed by decapitation 24 h after injection, and, at autopsy, the hind limbs from the left side were recovered, stripped of soft tissues, and fixed in 10% neutral formalin. The bones were subsequently decalcified in 10% EDTA (pH 6.5–7.0), embedded in paraffin and sectioned longitudinally through the knee joints at 5 μ on a rotary microtome. Serial sections were mounted on microscope slides; they were deparaffinized and autoradiographs were prepared by placing the slides in contact with Kodak NTA plates for 28 d at − 5 °C. After exposure, the slides were developed in Kodak D-19 for 5 min and fixed in acid fixer. The darkening of the developed images on the film was measured at the center of the epiphyseal cartilages (femurs and tibias) with a microscope-densitometer (30 μ aperture)-Brown Recorder combination, and the values were corrected for background.

Results. The microdensitometric readings indicated that the uptake of S^{35} in the epiphyseal cartilages of the femur and of the tibia were not significantly different. The femur and tibia data, therefore, have been combined in the Figure. Note that while the periodicity of the first day is essentially repeated in the second day, the values during the second day are somewhat higher from 15.00–18.00. This phenomenon, known as the 'serial effect' of HALBERG[4], is common to long-term periodicity experiments. Presumably, it is due to the fact that the animals were disturbed by the frequent entry into their quarters during the experimental period, and by handling during administration of the radiotracer. The uptake of S^{35} in the cartilages of the individual mice at each time varied by as much as a factor of 2. The mean value and the standard error of the mean together with the number of bones at each sacrifice time are plotted in the Figure. In 4 of the 65 mice in the experiment, the uptake of tracer in their cartilages proved to be either very high (day 1 in 1 mouse at 12.00 and 03.00; day 2 in 1 mouse at 09.00) or very low (day 2 in 1 mouse at 18.00); these points were excluded when they were in excess of 3 standard deviations from the mean. The graph clearly suggests that the amount of S^{35} incorporated (within chondrocytes) varies diurnally despite the great degree of individual variation. Maximum uptake was recorded during day 1 at 15.00 and from 15.00–18.00 during day 2–6 to 9 h after the onset of the photoperiod. Minimum uptake of S^{35} occurred throughout the dark environmental period (21.00–09.00). Statistically significant differences between the means of each group of femurs/tibias were found at 15.00 and 03.00 ($p > 0.01$).

Discussion. The results of this study in mice confirm our preliminary findings that the uptake of S^{35} in the growth cartilages is subject to diuranal variations[3]. The graph shows that S^{35} concentrations might be expected to increase in cartilage if the tracer were administered at 09.00, but that retention would not be large if radiosulfur were administered at 21.00. While the presence of clock hour changes in S^{35} uptake is consistent with other diurnal studies in cartilage[5,6], parallel biochemical investigations are required to prove that the clock hours of peak radiotracer uptake correspond to maximum chondroitin sulfate concentrations in chondrocytes. It has been shown in liver cells, for instance, that the peak hours of tritiated thymidine uptake and DNA content (determined spectrophoto-



A graph showing the 24 h pattern of S^{35}-sulfate uptake in the femurs and tibias of CF_1 albino mice injected at intervals of 3 h over a 48 h period. The horizontal bars represent the standard error of the means. Numerals enclosed in parentheses indicate the number of bones/group (double the number of mice).

[1] This work was performed under the auspices of the U.S. Atomic Energy Commission.

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metrically) are not necessarily coincident[7]. Despite species differences which obtain between laboratory rodents, the hours of maximal S^{35} uptake in the cartilages determined in this study appear to precede peak mitotic activity (rats) by 2–3 h[6]. This suggests that cartilage cells cannot pursue mitosis and mucopolysaccharide synthesis concurrently. It is equally probable that DNA cannot simultaneously support its own replication and the production of RNA[8]. Evidence from mouse studies, in which chondrocytes were labeled with tritiated thymidine[9], suggests that peak DNA synthesis (some time between 02.00 and 10.00) leads in phase both peak S^{35} uptake and mitosis. A somewhat similar multiparameter diurnal pattern has been recorded in different cell fractions from liver by BARNUM and his co-workers[10]. It is doubtful whether the periodicity in S^{35} retention can be explained by diurnal variations in dietary amino acid intake; one might expect dilution of intracellular pools of radiosulfur when mice are most actively feeding. Radioglycine experiments suggest, rather, that the diurnal period in the synthesis of the protein moiety of the cartilage ground substance is maintained even when animals were starved before study[11].

Résumé. Nous avons administré du soufre radioactif par voie i.p. à des souris, chaque groupe recevant l'injection à un moment différent de la journée (intervalle de 3 h entre les injections à 2 groupes successifs). L'analyse microdensitométrique d'autoradiographies du traceur dans les cartilages de conjugaison (fémurs, tibias) 24 h après l'injection a démontré que la rétention était la plus grande chez les souris qui avaient reçu l'injection entre 15 et 18 h.

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The Initiation of Contraction by Extracellular Calcium in the Smooth Muscle of the Guinea-Pig *Taenia coli*

It is generally accepted that the spike height is related functionally to the influx of the cation which carries inward positive charge. Contraction height is also related to the concentration of Ca^{++} injected into the muscle fibre[1]. In addition, HAGIWARA et al.[2] reported that Ca^{++} carries the charge during the rising phase of the action potential in barnacle muscle. The possibility of this Ca spike has also been reported in the smooth muscle of the guinea-pig *Taenia coli*[3,4]. The present experiments were performed to investigate the relation between the Ca spike and the twitch tension.

Taeniae coli of the guinea-pig were incubated in modified Krebs solution. Electrical activities were observed by means of sucrose-gap method and tensions were measured with a mechano-electronic transducer RCA 5734. Experiments were performed at low temperature (18 °C) to block spontaneous spike discharge, for the purpose of observing contraction as a single twitch. Single spike and twitch were evoked by supramaximal external stimulation. Tetrodotoxin (5×10^{-6} g/ml) was used to suppress Na spike activities. The toxin also blocked the inhibitory potential of the preparation by external stimulation[5].

Figure 1 shows that the amplitude of both action potential and contraction height varied with Ca^{++} concentration in the solution; the changes in amplitude were almost parallel. The minimal concentration for the generation of action potential and contraction varied from 10^{-6}–10^{-5} *M*. Dissociation of electrical and mechanical activities was not observed in any solution, regardless of difference in Ca^{++} concentration.

As these spike heights were measured by means of the sucrose-gap method, they represent the summation of the spike potential of different muscle cells.

The possibility exists that each cell generated an all-or-none spike. However, judging from the duration of the action potential, synchronization might well have occurred. Moreover, it is also known that the spike height,

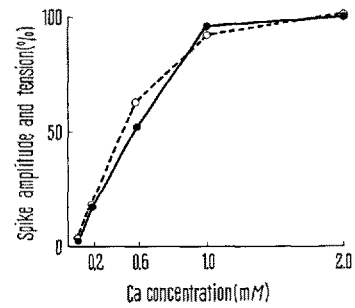

Fig. 1. The relation between spike height and twitch tension, and calcium concentration. Both spike heights and tensions were plotted against calcium concentrations as % of those observed in the solution containing 2 m*M* of calcium. Note that spike heights and contractions were parallel to each other relating to calcium concentrations. Closed circle, tension; open circle, spike amplitude.

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